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Elucidating the Immunoactivity of a Goat Serum Peptide

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ELUCIDATING THE IMMUNOACTIVITY OF A GOAT SERUM PEPTIDE

By

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The purpose of these studies was to determine if an immunomodulator was present in caprine serum. Controlled studies demonstrated that CSF-I, material fractionated from caprine serum possessed an immunomodulatory compound. Caprine serum was further fractionated into it s peptidic components and a small contaminant of immunoglobulin G and albumin (Caprine serum fraction - immunomodulator 2, or CSF-I2). This was refined to a three peptidic isolate collectively identified as tri-peptidic immunostimulant or TPI. CSF-I2 does not possess antibacterial capabilities (as typically characteristic of a cationic peptide or defensin), does not contain a level of endotoxin sufficient to promote a pyrogenic response, and its functional ability to improve animal survival after an infectious challenge does not reside with molecular weight components greater than 10 kilodaltons, effectively excluding the immunoglobins, albumin, cytokines, and collectins.

CSF-I2 was able to significantly reduce the mortality observed in chickens (from 80% to 13%) infected with *Pasteurella multocida* (Willeford *et al.*, 2000), in mice (from 83% to 13.3%) infected with *Salmonella typhimurium*, and in canines (from 50% to 9.8%) diagnosed with parvovirus. CSF-I2 may well prove to provide prophylactic and therapeutic health benefits to humans. CSF-I2 may effectively combat pathogenesis when used as either an adjunct with conventional therapy (e.g., antibiotics) or when provided as the primary medicant.

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LIST OF ABBREVIATIONS

ATCC	American Tissue Culture Collection
CPV	canine parvovirus
cfu	colony forming units
CSF-I	Caprine serum fraction - immunomodulator
CSF-I2	Caprine serum fraction - immunomodulator 2
IFN	interferon
IL	interleukin
i.p.	intraperitoneal
LPS	lipopolysaccharide
MIC	minimum inhibitory concentration
MM	molecular mass
MWCO	molecular weight cut-off
NK	natural killer
RP HPLC	reverse phase high performance liquid chromatography
sc	subcutaneous
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SPF	specific-pathogen-free

TNF	tumor necrosis factor
TPI	tri-peptidic immunostimulant

CHAPTER I

INTRODUCTION

The penicillin era was born in 1924 after a chance discovery by Alexander Fleming. Antibiotics quickly filled a void in medical science and are recognized to have saved countless lives. Man s dependence on antibiotics and the ability of bacteria to develop and disseminate antibiotic resistance through extra-chromosomal DNA may have now ushered in the end of the penicillin era. As a consequence, once-standard treatments for infections have become less reliable. The efficacy of last resort therapies like vancomycin can no longer be taken for granted (Roberts, 2001). Bacterial resistance has been reported even for recently implemented next generation antibiotics, such as the first oxazolidinone Linezolid (Tsiodras *et al.*, 2001).

The widespread prophylactic and therapeutic use of drugs has placed pathogenic microbes under constant antibiotic exposure, thus accelerating their need to adapt by generating resistance (Hancock, 1997; Aarestrup *et al.*, 1999; Virk and Steckelburg, 2000). The consequence of our cavalier attitude towards antibiotics may be grim. It has been predicted that within one or two generations, hospital wards will return to the overcrowded state that existed in the early 1900's when flu epidemics killed thousands. Agriculturally we can curtail the use of antibiotics to minimize disease related losses and

limit the amount of human exposure to antibiotic residue found in food products (Kogut, 2000) however may be too little too late. Bacteria unresponsive to available antibiotic therapy are becoming more common. Innovation has become a clarion call for developing new therapeutic regimes.

Since most living organisms are very similar at the molecular level, it is difficult to find substances that are lethal to certain organisms without being harmful to others. For this reason, there is a growing interest in discovering agents that augment one s innate immune defenses in order to prevent and treat infectious diseases (Rush, 2001). Innate immunity is mediated by cells, such as phagocytes and natural killer cells, circulating proteins (e.g., the complement system), and numerous antimicrobial peptides, which provide early host defense against infections before the development of an adaptive immune response. Innate immunity also stimulates adaptive immune responses (Abbas and Janeway, 2000).

Antimicrobial peptides are often associated with a class of peptides identified as cationic. Cationic peptides are lethal to a wide range of microorganisms, including gram-positive and gram-negative bacteria, fungi, parasites, and enveloped viruses because they induce leakage in membranes (Ganz, 1999). Cationic peptides can be defined as proteins with less than 100 amino acids (more typically less than 35) and a net positive charge of at least +2 (and usually more than +4) by virtue of their having an excess of lysine and arginine residues over acidic residues (Hancock, 1997; Hancock, 1999). Two shortcomings of these natural compounds, however, are that (1) very high peptide concentrations are needed for antimicrobial efficacy and (2) the difference in

toxicity (therapeutic index) between target and host cells is not sufficiently high (Maloy and Kari, 1995).

The primary goal of researching immunostimulation is to minimize reliance on antimicrobial drugs and associated issues of resistance and residue (Rush, 2001). Immunostimulant preparations produce nonantigen-specific enhancement of humoral and cell-mediated defense mechanisms (Tizard, 1993). It is believed that, through the use of immune regulating or immune modulating molecules, a state of general hyperactivity of the immune system can be induced which may be useful in combating and/or preventing a microbial infection. If such a non-specific immune response could be initiated at will, it could be utilized to either act alone or in conjunction with a conventional treatment directed towards the etiological agents. This could markedly impact both the agricultural, veterinary, and human medical communities.

Immunomodulators can be divided into three main groups: (a) immuno-suppressive agents; (b) immunostimulating agents (e.g., *bacillus* Calmette-Guérin vaccine); and (c) the remaining immunomodulators, which include biological response modifiers (e.g., colony stimulating factors, interleukins, interferons, and tumor necrosis factors) (Takx-Kohlen, 1992; Molloy *et al.*, 1993). Cytokines are soluble, low molecular weight polypeptides and glycopepetides produced by a broad range of cell types that have suppressive or enhancing effects on cellular proliferation, differentiation, activation, and motility. For the most part, they are not constitutively secreted, but are produced in response to stimulation by infectious agents or their derived products (e.g., endotoxin),

inflammatory mediators, mechanical injuries, and cytokines themselves (Kogut, 2000). An overview of some of the typical immunomodulatory agents is presented in Table 1.1.

The concept of immunostimulation originated in 1907, when William B. Coley noted spontaneous tumor regression in some patients after an episode of septicemia (Rush and Flaminio, 2000). In human and veterinary medicine, immunostimulant preparations are used primarily for treatment of chronic viral or bacterial infections. In some instances, immunostimulants have demonstrated efficacy as primary or adjunct treatment of neoplastic conditions (Rush, 2001). The proposed mechanism of action of nonspecific immunostimulant preparations is macrophage activation and subsequent release of cytokines that enhance the immune response (Rush and Flaminio, 2000). Prophylactic administration of immunostimulant preparations prior to pathogen exposure can decrease morbidity and mortality associated with acute infection (Rush, 2001).

In human medicine, immunomodulatory preparations have progressed from crude microbial, viral, plant, and thymic extracts to synthetic viral complexes and chemically defined drugs (recombinant cytokines). The crude extract preparations induce nonspecific immunomodulatory activity via macrophage activation. The benchmark of nonspecific immunomodulators is protection against a 50% lethal bacterial challenge in laboratory animals. The more sophisticated polyribonucleotide complexes are intended to mimic the interferon-inducing capacity of viruses. Newer generations of recombinant cytokines have selective effects on particular components of the immune system (Rush, 2001).

Because cells of the immune system and naturally occurring immunomodulators circulate through the blood and lymphatic system, serum is a logical place to look for immunomodulators. To date, only about half of the over 100 serum proteins have been isolated and characterized (de Gruyter, 1997), thus leaving ample opportunity for such a discovery. Caprine Serum Fraction - Immunomodulator (CSF-I) is a nonadjuvanted immunomodulator derived from goat serum. CSF-I remains ill-defined but is composed of a mixture of serum proteins and peptides. Field trials have shown efficacy of CSF-I against equine lower respiratory disease, a mixed etiological challenge (Hamm *et al.,* 2001). This suggests that CSF-I may possess a general non-specific immunomodulator, enabling recipients to combat a variety of stress challenges. However, no clinical trials had been performed.

The purpose of these studies was to determine if an immunomodulator was present in caprine serum by establishing its clinical efficacy in a murine model of salmonellosis. Further studies were performed in order to isolate and characterize the active agent and to elucidate its spectrum of efficacy and mode of action.

Table 1.1. Classification schema for immunotherapeutic drugs, adapted from Hadden and Smith, 1992 and Hadden, 1993.

Class	Example(s)	Biological Activity
Biologic agents		
Cytokines	Interferons	Antiviral, immunomodulatory, and antiproliferative activity
	Colony stimulating factors	Induce proliferation/differentiation of granulocyte progenitor cells
Fungal products	Glucans	Expand the reticuloendothelial system and activate macrophages
Thymic hormones	Thymulin, Thymopoietin	Modulate T cell function
Microbial products	*Propionbacterium acnes*	Stimulates macrophages, IL-1 and IFN production, and NK cells
	Mycobacterium	Macrophage activation and subsequent release of cytokines
Synthetic Immunostimulators		
Thymomimetics	Levamisole hydrochloride	Potentiates leukocyte stimulation by antigens, mitogens, etc.
Interferon inducers	Pyrimidinoles, Ampligen	Induce IFN, activate macrophages and NK cells
Muramyl dipeptide derivatives		Adjuvant activity

CHAPTER II

PROPHYLACTIC EFFECTS OF CAPRINE SERUM FACTOR (CSF-I) IN MICE INFECTED WITH *SALMONELLA TYPHIMURIUM*

INTRODUCTION

Caprine Serum Fraction - Immunomodulator (CSF-I) is marketed as a non-adjuvanted immunomodulator derived from goat serum. CSF-I is composed of a mixture of serum proteins and peptides and has a limited license for veterinary use (Ansley, 1993). Field trials have shown efficacy of CSF-I against bacterial, viral, and environmental stress challenges as found in equine lower respiratory disease (Hamm *et al.,* 2001), bovine shipping fever and respiratory disease, canine parvovirus and lymphoma, and ovine footrot (Ansley, 1993). The broad spectrum of efficacy suggests that CSF-I may possess a general non-specific immunomodulator, enabling recipients to combat a variety of stress challenges.

In mice, host-adapted salmonellae invade and multiply in the tissues of the reticuloendothelial system, with the severity and outcome of the disease dependent upon the infecting dose, the virulence of the bacterial strain, and the genetic background of the animal (Mastroeni *et al.*, 1994; 1999). In lethal infections, bacterial proliferation in the tissues progresses unrestrained until large bacterial numbers are reached. In sublethal

infections, survival requires an early host response that controls the growth of the organism in the tissues (Maskell *et al.*, 1987; Hormaeche *et al.*, 1990).

The purpose of this study was to assess whether CSF-I provides prophylactic benefit to mice systemically infected with *Salmonella typhimurium*. Six-week old, female Swiss Webster mice were injected i.p. with *Salmonella typhimurium* (~5.00 x 10^3 bacteria/ mouse) on day 0, effectively establishing an LD_{80} by day 8 post-challenge. Some of the mice infected with *S. typhimurium* were also previously given subcutaneous injections of CSF-I. Mortality was compared between mice given no CSF-I and those provided CSF-I at various periods prior to infection. Studies were performed in this manner to determine the optimal time of CSF-I administration and a CSF-I dose response profile. Spleens were examined for the presence of *S. typhimurium* so that the proliferation of pathogen could be compared among the groups, and blood samples collected during the course of this study were utilized for differential cell count analysis.

MATERIALS AND METHODS

Animal Care

Four-week old, female Swiss Webster mice were purchased from Charles River Laboratories (Wilmington, MA). The mice were acclimated for 2 weeks, during which time they were fed a standard maintenance diet, Laboratory Rodent Diet 5001 (PMI Feeds, Inc.), and watered *ad libitum*. Mice were group-housed (5 mice per cage) in plastic boxes bedded with wood shavings in an American Association for the Accreditation of Laboratory Animal Care (AAALAC)-accredited facility. Mice were

transferred to an isolation room immediately after inoculation with *S. typhimurium*. The isolation room was maintained at 20ºC in a controlled negative pressure environment on a 12 h lighting cycle. Animal care and use were in accordance with the policies of the Institutional Animal Care and Use Committee of Mississippi State University.

Bacteria

Salmonella typhimurium (ATCC 14028) was used as the challenge organism after passaging three times through a murine host with subsequent isolation and stored in phosphate buffered saline with 10% glycerol at -80ºC. This isolate was supplied from a stock culture from the Department of Biological Sciences (Mississippi State University) where it is maintained as a reference organism. Culture rehydration and cryoprotective maintenance conditions have been described previously (Darnell *et al.*, 1987).

Experimental Design

Mice comprising the control (positive control) and treated populations were injected intraperitoneally (i.p.) with 0.1 ml of *Salmonella typhimurium* (~5 x 10^3 bacteria/mouse) on day 0. Unless stated otherwise, treated mice were given a 0.1 ml subcutaneous (sc) injection of CSF-I (Colorado Serum Co. - 30 mg protein/ml) at the time designated by the experimental protocol, while control mice received a placebo of physiological saline. Negative control mice were sham handled in a similar manner to the control and treated populations to evaluate the influence of non-experimental parameters on mortality. Mice were housed five per cage and a minimum of six cages

were used per treatment group. Mice were monitored three times daily and mortality recorded until 80% of the control mice died or for a maximum of two weeks.

Systemic Proliferation of Salmonella

Ten mice that had neither been treated with CSF-I nor exposed to *S. typhimurium* were euthanized by CO_2 asphyxiation, their spleens harvested, and blood drawn from the inferior vena cava for differential cell count analysis. Each spleen was weighed, diluted 10-fold with sterile water, and homogenized. Aliquots of homogenate were plated on MacConkey agar, a medium selective for gram negative bacteria, and cultured overnight at 37°C. The number of *S. typhimurium* cfu were visually identified and the baseline value determined per g of spleen. Approximately 50 l of blood was streaked across a microscope slide, air dried, stained with Wright s stain (Fisher Scientific Company, Houston, TX), and a differential cell count analysis performed.

Four treatment groups were established: negative control (mice which received neither the bacterial challenge or CSF-I), positive control (mice which received the bacterial challenge but no CSF-I), CSF-I treated mice (mice which received both the bacterial challenge and CSF-I), and the treatment control (mice which received CSF-I but no bacterial challenge). Each group was formed by randomly assigning a housing cage with five mice to a treatment regime until 14 cages were enrolled in the regime.

CSF-I-treated mice were injected sc with 0.1 ml CSF-I on day -1. The positive control and CSF-I treated populations were challenged with *S. typhimurium* on day 0. On days 2 through 7, a minimum of five mice from each group was selected randomly and

their spleens harvested. Blood was drawn from all treatment groups for analysis as described above.

Differential Cell Counts

Differential cell counts were determined on blood smears stained with Wright s stain. The lymphocytes, heterophils, monocytes, eosinophils, and basophils were identified by the morphological characteristics described by Lucas and Jamroz (1961). Baseline values were derived from averaging ten negative control mice. Daily values for each treatment regime were assessed by averaging a minimum of 5 observations.

Salmonella typhimurium Microagglutination Assay

A *Salmonella typhimurium* microagglutination test along with its positive and negative control standards was purchased from the Center for Veterinary Biologics and National Veterinary Services Laboratories, United States Department of Agriculture (Ames, IA) and performed according to the testing protocol supplied. The titer of *Salmonella* antibody was determined for CSF-I (Colorado Serum Company) to help ascertain whether a sufficient titer was present to be responsible for the observed prophylactic benefits.

The prophylactic benefit derived from administration of purified goat IgG (Sigma Chemical Company) was ascertained in accordance with the murine experimental model described above. Mortality observed in the population of control mice was compared to mice which received purified goat IgG (2 mg/mouse).

Limbulus amebocyte Lysate (LAL) Endotoxin Quantitation Assay

The standardized endotoxic activity of CSF-I was determined by using an LAL gel clot assay by Associates of Cape Cod (Woods Hole, MA). The threshold sensitivity of reaction was 0.03 endotoxic unit (EU) per ml.

Statistical Analysis

All experiments were arranged in a completely randomized design. Data demonstrating cumulative mortality were analyzed using one way analysis of variance (ANOVA) with the general linear models procedure (Proc GLM) and the means separated by Fisher s projected LSD procedure (SAS Institute Inc., 1997). A p value less than 0.05 was considered significant.

RESULTS

Salmonella typhimurium Microagglutination Assay

The *S. typhimurium* microagglutination assay showed a positive reaction for up to a 1:8 CSF-I dilution in replicate testing (n = 3).

LAL Endotoxin Quantitation Assay

CSF-I had an endotoxin level of 0.31 EU/ml (0.01 EU/mg).

Multiple Administrations of CSF-I

Onset of mortality occurred approximately 4 days post-challenge. It was of interest to determine whether the survival rate could be improved with supplemental administrations of CSF-I. Control mice were compared to two treated groups: one which received CSF-I only on day 0 and another which received CSF-I on days 0, 1, and 4 (Figure 2.1). Both treated groups had significantly fewer deaths than the control population between days 5 and 8. There was no significant difference in prophylactic benefit between the populations which received the single or multiple administrations of CSF-I. On day 7, the day 0 and days 0, 1, and 4 treated groups had a mortality of 17% and 14%, respectively.

Optimal Time of CSF-I Administration

A single dose of CSF-I was administered at various times prior to challenge to assess persistence of positive effects and to ascertain the optimal time of administration with regard to the *S. typhimurium* challenge model. CSF-I was administered on either day -4, -2, -1, or coincident with the challenge on day 0 (Figure 2.2). Three to four days were usually required before deaths were observed in control populations of female Swiss Webster mice challenged with *S. typhimurium* (~5 x 10^3 bacteria/mouse). A rapid rise in death ensued with ~80% mortality occurring 1 week post-challenge and ~90% mortality on day 8. Mice treated with CSF-I four days prior to challenge showed no significant difference from this pattern. Prophylactic benefit, however, was observed if CSF-I was given on either day -2 or 0. One week post-challenge, the control population

presented 83.3% mortality, while groups that received CSF-I on either day -2 or 0 had percent mortalities of 13.3 and 16.7, respectively. On day 8 these values rose to 90 (control), 36.7 (day -2), and 23.3 percent (day 0). Mice treated one day prior to challenge had the least number of deaths. Only 10% of this treated group were dead at days 7 and 8 post-challenge. Beginning with day 5 post-challenge (when mortality was established in the control population) there was a statistically significant difference between the control group and each of the day -2, -1, and 0 CSF-I treated groups. The day -1 treatment group was also significantly different from the day 0 and day -2 CSF-I populations.

Dose Response to CSF-I

A dose response study was performed in order to determine the optimal amount of CSF-I to administer for prevention of mortality (Figure 2.3). CSF-I was prepared so that a 0.10 to 0.25 ml i.p. injection would deliver either 0.3, 1.5, 3.0, or 7.5 mg CSF-I. All injections of CSF-I were given on day -1, as this was shown by the time course study to produce the greatest prophylactic benefit. On day 7, all doses were significantly different ($p < 0.05$) from the control mice except for the 0.3 mg CSF-I dosage. Seventy-seven percent of the control population died by day 7, while with respect to increasing amounts of CSF-I, 60, 33, 27, and 17% had died in these treated groups.

Systemic Proliferation of Salmonella

A 6 to 7 thousand fold increase in *S. typhimurium* was found in the spleens of control mice 2 to 3 days after pathogenic challenge (Table 2.1). Spleens were friable by day 3 and numerous grey white lesions visible. The proliferation of *S. typhimurium* went seemingly unchecked. There was an approximate 50,000-fold increase by day 4 which continued to escalate to a 2-3 million fold increase over the following three days. Mice treated with a single dose of CSF-I one day prior to challenge had no increase in *S. typhimurium* by day 2, and had less than a 100 fold increase on days 3 and 4. Treated mice retained a significant difference in *S. typhimurium* population on day 5 (over 700 fold less than control mice) but this margin narrowed to only a 5 fold difference by day 7.

Differential Cell Counts

A significant decrease in lymphocytes between positive control and baseline mice was noted beginning on day 3 and continued throughout the study. However, no significant difference was noted between CSF-I treated and baseline mice until day 5 (Figure 2.4a). On day 7, there was a 67% decrease in the percent lymphocyte count of positive control mice, while CSF-I treated mice only showed a decrease of 26%. There was no significant difference on any day between any treatment regime group and baseline mice for eosinophil or basophil counts. Further, analyses of the negative control and treatment control samples showed that none of the blood parameters tested were significantly different from those of the established baseline throughout the observation period.

Control mice showed a significant increase in percent neutrophil composition by day 2. A significant increase in both neutrophils (Figure 2.4b) and monocytes (Figure 2.4c) was noted in control mice by day 4 (269% and 279%, respectively). These values continued to increase throughout the observation period culminating at day 7 (461% and 369%, respectively). During this seven day period, neutrophil values for CSF-I treated mice increased 206%, and at no time were the monocyte counts significantly different from baseline values. Positive control mice also had a much greater percent of neutrophils showing toxic changes as evidenced by their foamy, vacuolated cytoplasm (73% versus 7% for CSF-I treated mice).

DISCUSSION

Post-operative patients and individuals with auto-immune dysfunction have been treated therapeutically with -globulin (Duswald *et al.*, 1980; Berkman *et al.*, 1990), administration which has also proved efficacious when used to treat the auto-immune disorders of antiphospholipid syndrome and systemic lupus erythmatosus developed in murine experimental models (Bakimer *et al.*, 1993; Krause *et al.*, 1995). CSF-I has a significant globulin presence and tested positive for *Salmonella* IgG up to 8-fold dilution. When purified goat IgG was administered as the sole prophylactic agent to combat salmonellosis, the mice responded in a manner consistent with control mice. While globulin is a functional participant of the immune system and has been shown to offer some therapeutic benefit, no mechanistic role could be ascribed to its administration in the response precipitated by CSF-I as observed in this salmonellosis model.

An inoculum s titer of pathogenic organisms greatly influences whether a lethal challenge develops. Host ability to quickly reduce the number of infecting organisms helps prevent the inevitable exponential growth phase from proceeding in an unrestrained manner, an essential element for host survival (Mastroeni *et al.*, 1999). Anti-microbial peptides such as defensins, found in plasma often bound to plasma proteins, help serve this role (Panyutich *et al.*, 1993). Defensins could easily be concentrated during the ammonium sulfate fractionation procedure used to manufacture CSF-I. Caprine serum and the large (10 kDa) and small (10 kDa) molecular weight material fractionated from it by size filtration were screened for bactericidal activity against a series of gram positive and gram negative bacteria including *Salmonella typhimurium* using disk agar diffusion assays (Willeford *et al.*, 2000). Bactericidal activity was not found in any of these products and therefore it is logical to conclude that CSF-I s prophylactic benefit is not a function of a typical defensin-like action.

The systemic nature of the *S. typhimurium* infection was monitored by determining its proliferation in splenic tissue and effects on the leukocyte profile. The onset of mortality was coincident with a bacterial count of approximately 10^6 cfu/g spleen. Control mice reached this level 4 days post-challenge, but CSF-I treatment helped abate pathogenic proliferation, preventing a lethal level from being reached until day 6. The delay/reduction in mortality observed in treated populations of mice may have resulted from CSF-I s ability to ready or stimulate the host s immune system for action. Maximal response occurred when CSF-I was administered 1 day prior to challenge, but significant benefit was observed when CSF-I was given either 2 days

before challenge or coincident with the challenge. Clearly, benefit did not extend beyond 4 days in the lethal model used here.

It is also apparent that there is an upper level to the ability of CSF-I to stimulate the immune system. No significant difference was observed 8 days after challenge in mice which received either the 3 or 7.5 mg dose of CSF-I one day prior to challenge. The dose response curve had plateaued. In addition, no benefit (as measured by survivability) was noted in mice which received several booster doses of CSF-I. This may be because the amount of CSF-I used in this experiment generated a near maximal activation of the immune response which then persisted for several days. An analogous dose response profile was observed when CSF-I was used to therapeutically treat horses suffering from lower respiratory disease (Hamm *et al.,* 2001)

Microbial infection alters the normal leukocyte profile to reflect the adaptive needs of the host. Neutrophils are rapidly deployed to the infection site followed by monocyte accumulation to phagocytose pathogens (Tizard, 1996). The stress response initiated by microbial infection therefore consists partially of neutrophilia, monocytosis, lymphopenia and inflammation (Zinkl, 1981; Latimer and Rakich, 1989; Maxwell, 1993). The leukocyte profile revealed by differential cell count analysis showed that control mice experience significant neutrophilia by day 2, monocytosis by day 4 and lymphopenia by day 3. Monocytosis was not evident in CSF-I treated mice throughout the 7 day observation period and significant neutrophilia and lymphopenia were each delayed by two additional days. These observations correlate with the bacterial proliferation data and mortality response. Treated mice were better able to respond to

the challenge permitting the host to mount a successful defense and clear the organism from its system. While the leukocyte profile of mice solely treated with CSF-I was unchanged, the recipient s state of readiness may have been heightened. Upon a recognized challenge (e.g., LPS stimulation) the host s immune system may respond more rapidly and vigorously.

Sublethal infections of *Salmonella* may proceed in four distinct phases: (i) an early inactivation of the challenge in the first 24 h; (ii) exponential growth; (iii) the plateau phase; and (iv) T-cell dependent bacterial clearance (Hormaeche, 1979; Hormaeche *et al.*, 1985). The studies reported here suggest that CSF-I can exert influence prior to activation of the adaptive immune response. It is possible that CSF-I may directly modulate the bactericidal activities of macrophages harboring *Salmonella* by activating NK cells - which are known to participate in resistance against microbial infection (Timonen, 1997), or possibly through modulation of macrophage function through natural killer cell-derived cytokines such as IFN- (Kagaya *et al.*, 1989; Ramarathinam *et al.*, 1993).

The search for agents which potentiate the immune response is a driving force in drug research. Use of immune regulating or immune modulating molecules should induce a state of general hyperactivity of the immune system which should precipitate and/or enhance the prophylactic response to immune challenges such as pathogenic infection. If such a non-specific immune response could be initiated at will, it could be utilized to either act alone or in concert with conventional treatments directed towards etiological agents. CSF-I appears to possess an immunomodulatory compound. This

agent does not appear to derive its prophylactic benefit from direct bactericidal activity or the direct action of administered globulin. It is unclear whether administration of CSF-I affects the ability of the host to kill the bacteria, control their subsequent rate of growth, or both. CSF-I, however, influences in a positive way the ability of the host to withstand and survive a challenge of an infectious agent. Further investigation is required to determine the nature of the immune-regulatory compound and elucidate its mode of action.

Table 2.1. Systemic proliferation of *Salmonella typhimurium*[a]

Treatment	*S. typhimurium* cfu/g of spleen (x 10^5)					
	Day after *Salmonella* Inoculation					
	2	3	4	5	6	7
Control	3.7 ± 0.8[b]	3.2 ± 0.5[b]	24 ± 2[b]	2000 ± 100[b]	890 ± 100[b]	1400 ± 200[b]
CSF-I	0.0005[c]	0.04 ± 0.03[c]	0.02 ± 0.02[c]	2.8 ± 0.7[c]	45 ± 6[b]	300 ± 120[b]

[a]Control mice were administered 0.1 ml (5 x 10^3 cfu) of *S. typhimurium* i.p. on day 0, while treated mice received both *S. typhimurium* on day 0 and a 0.1 ml sc injection of CSF-I (3 mg) on day -1. Spleens were harvested on days 2 through 7, homogenized and assessed for *S. typhimurium*. Each data point represents the average bacterial count/g of spleen ± SEM (n 5). A minimal cfu value of 100 was required to be detectable. *S. typhimurium* were not detected in baseline mice (n = 10) or on day 2 of the CSF-I treated population. A value of 50 was assigned to these populations in order to permit comparisons between groups.

[b-c]Means within a column lacking a common superscript differ ($p < 0.05$).

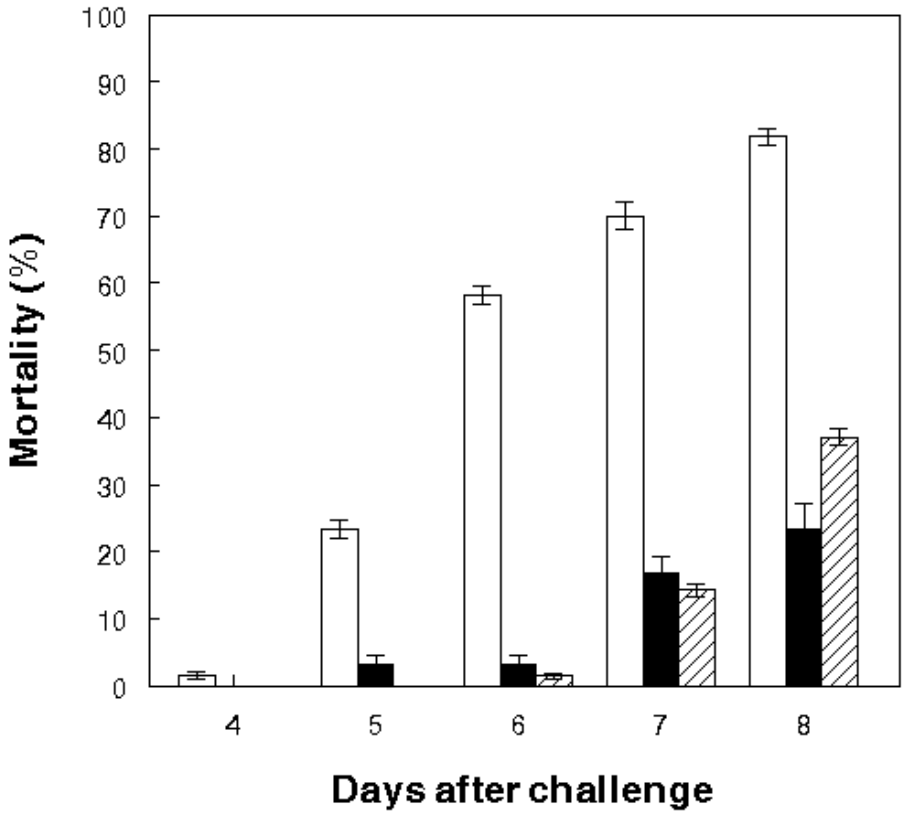

Figure 2.1. Effect of post-challenge administration of CSF-I.

Control mice were administered 0.1 ml (~5 x 10^3 cfu) of *S. typhimurium* i.p. on day 0 (open bar), while treated mice received both *S. typhimurium* on day 0 and a 0.1 ml sc injection of CSF-I (3 mg) on either day 0 (closed bar), or on days 0, 1, and 4 (hatched bar). Each data point represents the average cumulative mortality (n = 6) per cage of 5 mice.

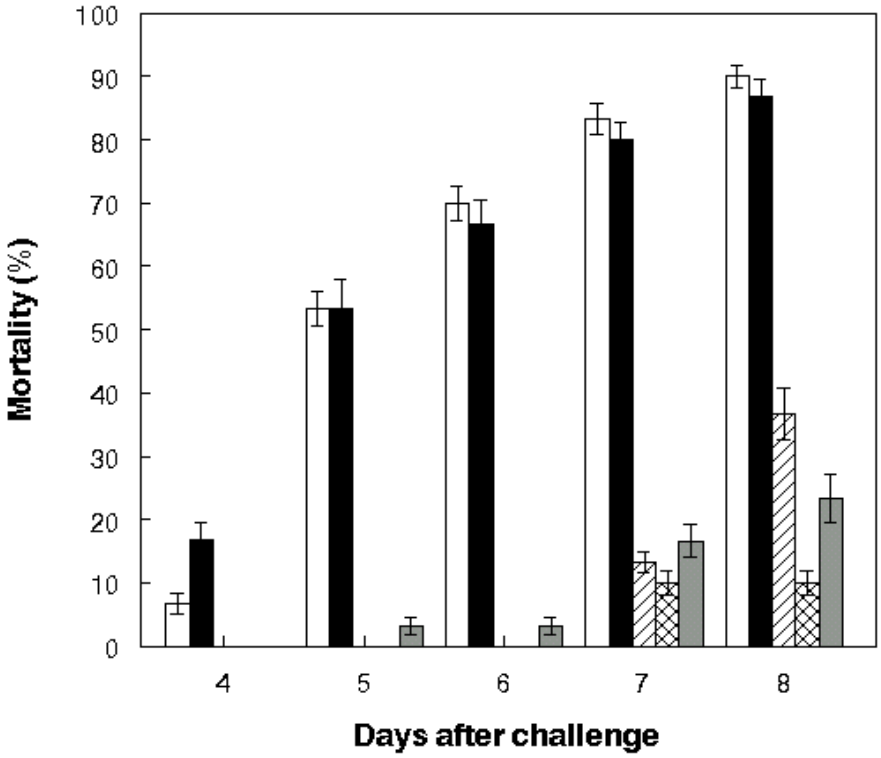

Figure 2.2. Optimal time of CSF-I administration (time course profile).

Control mice were administered 0.1 ml (~5 x 10^3 cfu) of *S. typhimurium* i.p. on day 0 (open bar), while treated mice received both *S. typhimurium* on day 0 and a 0.1 ml sc injection of CSF-I (3 mg) either on day -4 (closed bar), day -2 (hatched bar), day -1 (cross-hatched bar), or day 0 (shaded bar). Each data point represents the average cumulative mortality (n = 6) per cage of 5 mice.

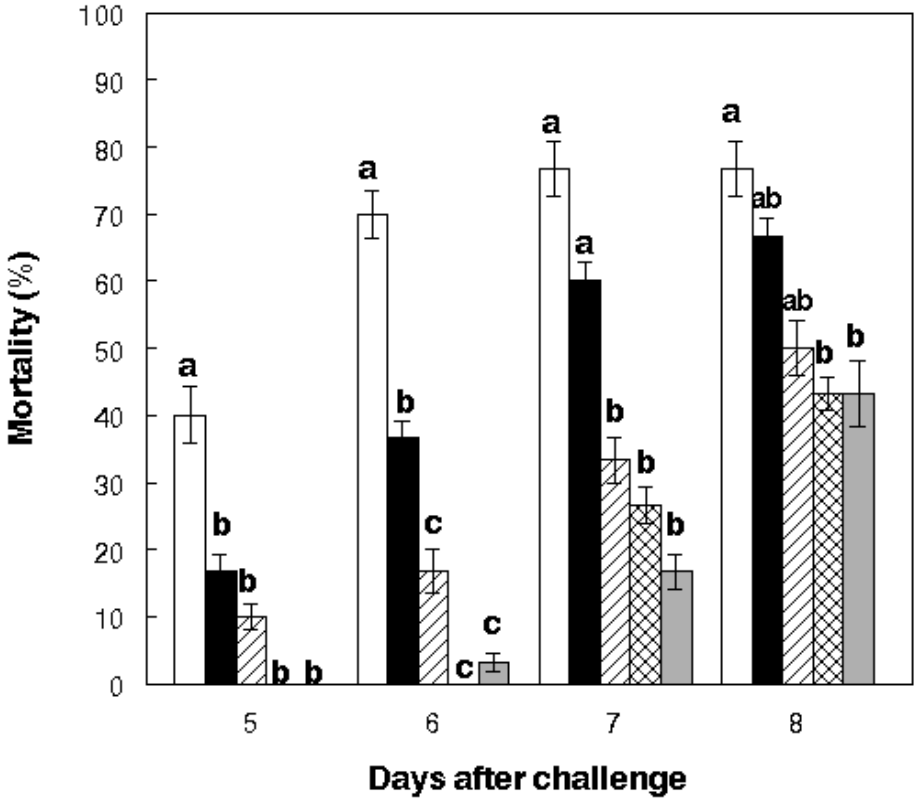

Figure 2.3. CSF-I dose response profile.

Control mice were administered 0.1 ml (~5 x 10^3 cfu) of *S. typhimurium* i.p. on day 0 (open bar), while treated mice received both *S. typhimurium* on day 0 and a sc injection of either 0.3 mg (closed bar), 1.5 mg (hatched bar), 3.0 mg (cross-hatched bar), or 7.5 mg (shaded bar) CSF-I. Each data point represents the average cumulative mortality (n = 6) per cage of 5 mice. Means with no common letter differ significantly ($p < 0.05$).

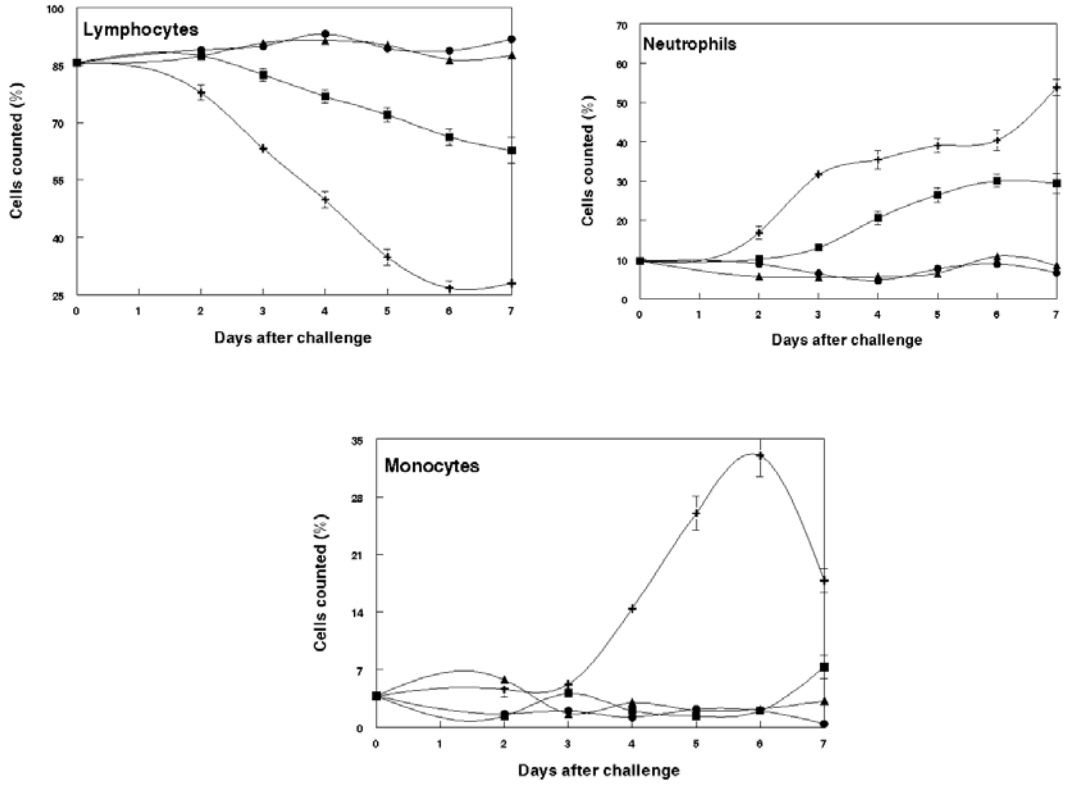

Figure 2.4. A comparison of differential cell counts between CSF-I treated and control mice after challenge with *Salmonella typhimurium*.

Ten mice that had been neither treated with CSF-I nor exposed to *S. typhimurium* were euthanized and approximately 50 l of blood was streaked across a slide for differential cell count analysis. The cellular identification of 100 leukocytes was performed based on morphological characteristics as described by Lucas and Jamroz (1961). Control (+) and CSF-I ( ) treated mice were prepared in accordance with the experimental design in Materials and Methods, as were the negative control ( ) and treatment control ( ) mice. On days 2 through 7, a minimum of five mice from each group was randomly selected and blood drawn for differential cell count analysis. The percent cell type ± SEM was determined for a) lymphocytes, b) neutrophils, and c) monocytes.

CHAPTER III

REDUCTION IN LAYER MORTALITY BY A CAPRINE SERUM FRACTION

INTRODUCTION

Discovering agents which potentiate the immune response is a driving force in drug research. Cytokines and cationic peptides are two classes of relatively low molecular weight compounds which have shown promise in this area of research. At least nine immuno-defense peptide products are already on the market with annual sales of over $4 billion (Latham, 1999).

Interleukin-1 (IL-1), tumor necrosis factor- (TNF-), and interferon (IFN) are three cytokines which participate in the immune response. IL-1 is involved in the host s response to antigenic challenge and tissue injury, and has been shown to increase the resistance of mice to pathogenic organisms such as *Listeria*, *Escherichia coli*, and *Candida albicans* (Czuprynski and Brown, 1987; Cross *et al.*, 1989; Pecyk *et al.*, 1989). TNF- and -IFN were able to increase the resistance of mice to *Salmonella typhimurium* (Morrissey and Charrier, 1994). Human IFN- s have potent antiviral and antiproliferative activities, and are currently being utilized as antiviral or anticancer therapeutic agents (Chang *et al.*, 1999).

Some cationic peptides have also been observed to act as an immunostimulant. A decameric peptide was shown to impede the growth and spreading of established tumors (Folkman, 1999). Other peptides promote antibacterial, antifungal, antiviral, and even wound healing properties (Hancock, 1999; Mizuno *et al.*, 1995; Sanglier *et al.*, 1993). It is believed that these defense peptides are more general in their actions than antibodies, and as such, have a broader range of activity (Hancock, 1999).

Because cells of the immune system circulate through the blood and lymphatic system, serum is a logical place to look for immunomodulators. To date, about half of the over 100 serum proteins have been isolated and characterized (de Gruyter, 1997). Caprine Serum Fraction - Immunomodulator (CSF-I, US Patent 5,219,578.) is a nonadjuvanted immunomodulator derived from goat serum. CSF-I remains ill-defined but is composed of a mixture of serum proteins and peptides.

It is important to develop rapid and reliable screening assays for the isolation and characterization of the components in CSF-I responsible for potentiating the immune response. TNF- bioassays proved to be unreliable. Along with the pursuit of effective screening assays, the development of reliable animal models by which to confirm the presence of the compound of interest continues. This study presents an avian model which can be performed reliably over a two to seven day period. This is also the first study to show that material fractionated from goat serum helps retard pathogenesis due to bacterial infection in a non-mammal, supporting the belief that CSF-I is a nonspecific immunomodulator.

MATERIALS AND METHODS

Animal Husbandry

Fertile eggs obtained from specific-pathogen-free (SPF) Leghorn chickens (HY-Vac Laboratory Eggs, Co., Adel, IA, 50003) were incubated under standard conditions. Upon hatch, 120 chickens were equally and randomly assigned to each of 12 Horsfall-Bauer isolation units. Birds were raised in this manner until they were 5 weeks of age, at which time the *Pasteurella* challenge was administered. Bird density was 360 cm^2 per bird at 5 weeks. All chickens had *ad libitum* access to feed and water. Birds were fed a standard pullet starter diet that met or exceeded National Research Council (1994) recommendations. The isolation room was a negative pressure environment maintained at 25°C with a 12 h light:12 h darkness cycle.

Bacteria

Pasteurella multocida X-73 (serotype 1) was obtained from the National Animal Disease Center, Ames, Iowa. *P. multocida* was grown in brain-heart infusion (BHI) broth (Difco Laboratories, Detroit, MI) at 37°C. After 16 h incubation, 1 ml of culture was transferred to 100 ml of fresh BHI broth and incubated at 37°C for 4 h. The bacterial culture was serially diluted with sterile water in 10-fold increments. Each dilution was plated on blood agar plates and the colonies counted after 24 h incubation to determine the number of cfu (Wang and Glisson, 1994).

Pasteurella multocida P-1581, *Pasteurella multocida* ATCC 11039, *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas aeruginosa* PAO1, *Escherichia*

coli ATCC 25922, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Staphylococcus aureus* T-5706, and *Bacillus subtilis* were supplied from stock cultures by Dr. F. R. Champlin (Department of Biological Sciences, Mississippi State University) where they are maintained as reference organisms. Culture rehydration and cryoprotective maintenance conditions have been described previously (Darnell *et al.*, 1987).

Preparation of Caprine serum fraction - immunomodulator 2 (CSF-I2)

CSF-I2 was fractionated from goat serum by collecting the material which flowed through a dialysis membrane (Spectrum Laboratories, Inc., Laguna Hills, CA) with a molecular weight cut-off of 6-8 kDa. This low molecular weight material was lyophilized to a powder and stored at -70ºC until reconstituted with water for use. Powdered CSF-I2 was 37.5% proteinaceous based on its nitrogen content.

Susceptibility Assays

Caprine serum and its high (8 kDa) and low molecular weight (8 kDa) subfractions (each at a protein concentration of 20 mg/mL) were assessed for antimicrobial activity against both gram positive and gram negative bacteria by performing disk agar diffusion assays as described in Hart and Champlin (1988). Antibiotic minimum inhibitory concentrations (MICs) were determined in Mueller-Hinton broth (Difco Laboratories, Detroit, MI) using the broth dilution method described previously (Darnell *et al.*, 1987). Caprine serum and its dialysate (8 kDa) were unable

to inhibit bacterial growth. CSF-I2, material presumed to contain small molecular weight immunomodulatory agents, also failed to inhibit the growth of all test bacteria, including two serotypes of *Pasteurella* (Table 3.1).

Bird Treatment

Two of 12 housing units were utilized as stress control pens. Birds assigned to these units did not receive a bacterial challenge or CSF-I2, but were sham handled in a manner consistent with all other birds. All inoculated birds received 18-30 cfu of *Pasteurella multocida* (X-73 strain, serotype 1) on day 0. CSF-I2 and sham treatments, as well as bacterial challenges, were administered as 0.5 ml intramuscular (i.m.) injections.

Control birds housed in five of the remaining ten units received *Pasteurella* and sham treatment in a manner consistent with CSF-I2 treated birds. In experiment 1, treated birds were given 0.5 ml of a 10 mg/ml CSF-I2 solution (5 mg) on days -1 and 0. In the second experiment the treated birds received 0.5 ml of a 20 mg/ml CSF-I2 solution (10 mg) on days -1 and 0. Treated birds in the third experiment received 0.5 ml of a 20 mg/ml CSF-I2 solution (10 mg) on days -1, 0, and +1. The number of deaths were recorded and dead birds were removed from each unit at the same time each day. Each experiment utilized a seven day trial period wherein the average cumulative mortality per day was compared among the control and treated groups.

Flow Cytometry

Birds used in Experiment three were banded for identification. Five birds were selected at random from both the control and CSF-I2 treated populations. Two ml of blood was drawn from a wing vein and immediately processed for CD4 and CD8 counts. Blood was drawn 1 week prior to the *Pasteurella* challenge in order to establish a representative baseline count. Blood was also collected on day 1 in order to assess how the challenge and CSF-I2 treatment may alter these immunological parameters.

The CD4 and CD8 lymphocyte subset percentages in the peripheral blood were enumerated using a modification of a previously published method (Ainsworth *et al*., 1990). Briefly, Ficoll-Paque isolated peripheral blood leukocytes were incubated with FITC-conjugated mouse anti-chicken CD4 or RPE-conjugated mouse anti-chicken CD8, or the appropriate isotype-matched control conjugates (Southern Biotechnology Associates, Inc., Birmingham, AL). Modifications to the procedure included a reduction of the incubation times to 5 min and analysis of samples using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA).

Differential Cell Counts

A total of 200 nonerythroid, nonthrombocytic leukocytes were counted on duplicate blood smears stained with Wright s stain (Sigma Chemical Co., St. Louis, MO). The lymphocytes, heterophils, monocytes, eosinophils, and basophils were identified by the morphological characteristics described by Lucas and Jamroz (1961), and the relative percentage of each identified cell type reported.

Statistical Analysis

All experimental protocols required a completely randomized design. Data demonstrating cumulative mortality and diagnostic blood parameters were analyzed using one way analysis of variance with the general linear models procedure (Proc GLM) and the means separated by Fisher s projected LSD procedure (SAS Institute Inc., 1997). A probability less than 0.05 had to be reached in order to be considered significant.

RESULTS

Birds receiving the 5 mg CSF-I2 treatment regime on days -1 and 0 demonstrated significantly less mortality than inoculated controls throughout the study period (Figure 3.1). However, the greatest difference was observed within the first 48 h after challenge. During this 2 day period, the control group receiving 30 cfu of *P. multocida* displayed the greatest mortality. Forty-nine percent of the control population were dead after 24 h and 91% were dead by day 2. The 5 mg CSF-I2 treatment regime, however, significantly retarded pathogenesis of the *Pasteurella* infection. Mortality in CSF-I2 treated birds were 6 and 56% on days 1 and 2, respectively. Mortality gradually increased to 78% over the following 5 days of the study period.

Pathogenesis was retarded further when the CSF-I2 dose regime was doubled to 10 mg (Figure 3.2). A *Pasteurella* challenge of 18 cfu caused rapid mortality in the control population (60% and 80% for days 1 and 2, respectively). Mortality in the corresponding CSF-I2 treated group was only 0 and 13% on days 1 and 2, respectively. Mortality on day 7 had risen to 33%, which was significantly less than that of the control

population (83%), and was less than that observed when the 5 mg CSF-I2 treatment regime was used.

The 10 mg CSF-I2 treatment regime was extended to include an additional delivery dose on day 1 (after the daily observation). Twenty-eight cfu of *Pasteurella* were used to initiate pathogenesis. After 24 h, mortality in the CSF-I2 treated population was significantly less than that of the control population (12% and 77%, respectively; Figure 3.3). Mortality on days 2 and 7 in CSF-I2 treated birds (38% and 56%, respectively) were consistently lower than that found in the corresponding untreated control population (94% and 97%, respectively).

Both treatment regimes utilizing 10 mg of CSF-I2 allowed birds to survive the bacterial challenge better than the 5 mg CSF-I2 treatment regime (Figure 3.4). There was no evidence, however, which showed that the additional administration of CSF-I2 on day 1 initiated a significantly better response than the 2 day dose regime of 10 mg CSF-I2.

The blood levels of CD4 and CD8 cells in unchallenged SPF chickens of Trial 3 were 18 and 26%, respectively. The microbial challenge caused the day 1 CD4 cell count to decrease to 10% ($p < 0.05$) while birds treated with CSF-I2 expressed a CD4 cell count of 21% (not significant). No appreciable changes in CD8 levels occurred due to CSF-I2 treatment.

The highest rate of mortality due to *Pasteurella* pathogenesis occurred within the first 24 h. Differential cell counts were taken for control and CSF-I2 treated birds at 24 h and compared to baseline values (Table 3.2). Control birds showed a significant drop in

lymphocytes and increase in heterophils. CSF-I2 treated birds showed the same trends in lymphocytes and heterophils; however, the changes were not as severe. Although the differences were significant, it is unknown as to how indicative these values are because of the small sample size and high death rate, which made it impractical to follow the same birds throughout the study. There were no significant differences in monocytes, basophils, and eosinophils between CSF-I2 treated and control birds.

DISCUSSION

Agents which retard pathogenesis may enable a host to mount a successful defense to challenges of the immune system. These agents can provide specific protection (i.e., in the form of antibodies) or be general in nature and enhance overall immunocompetence. Cytokines and cationic peptides are two such classes of non-specific defense agents. CSF-I is a non-adjuvanted immunomodulator derived from goat serum containing a mixture of serum proteins and peptides. Identifying the immuno-active components in CSF-I (and therefore goat serum) requires a bioassay. The *Pasteurella* challenge model utilized in this study was shown to be an effective bioassay in chickens.

Immunoglobin and albumin are the two most abundant serum proteins. The size fractionation procedure employed removed proteins with a molecular weight greater than 8 kDa. Therefore, it excluded immunoglobin, albumin, and most cytokines. This composite of low molecular weight material (CSF-I2) was unable to inhibit growth of gram-negative and gram-positive bacteria. The minimum inhibitory concentration (MIC)

for a cationic peptide is usually in the range of 1 to 8 g/mL (Hancock, 1997). Two serotypes of *Pasteurella* showed no zone of growth inhibition when CSF-I2 was tested at 20 mg/mL, over 2,500 times the upper MIC range recognized for cationic peptides.

SPF layer chickens experience a severe rate of mortality when challenged by low doses (18 to 30 cfu) of *P. multocida* X-73 (serotype 1). CSF-I2 was, however, able to significantly retard *Pasteurella* pathogenesis and promote higher survivability in SPF chickens. Three treatment regimes of CSF-I2 were examined in this study: 5 mg CSF-I2 (i.m., days -1 and 0), 10 mg CSF-I2 (i.m., days -1 and 0), and 10 mg CSF-I2 (i.m., days -1, 0, and 1). All treatments effectively reduced mortality through 1 week post-challenge. The 10 mg CSF-I2 dose regimes, however, clearly performed better than the 5 mg CSF-I2 dose regime. Variances in experimental parameters make a rigorous statistical comparison between the two 10 mg CSF-I2 dose regimes impossible. However, there was no evidence that day 1 administration of CSF-I2 improved survivability. This may be because the amount of CSF-I2 used on days -1 and 0 had generated a near maximal activation of the immune response which then persisted for a period of time.

Heterophils are the first line of defense. They are the earliest phagocytic cells to be recruited to a site of infection, and usually only survive for a few hours after leaving the bone marrow (Bancroft *et al.*, 1997). The elevated heterophil count found in control birds may indicate a need for their continued release as these birds were unable to clear the pathogen, evidenced by the high rate of mortality. Treated birds, however, expressed a lower heterophil count, indicating that they were better able to combat the infection.

CD4s are associated with Helper T cells, while CD8s are associated with killer cells, which kill other cells and may suppress the immune response (Tizard, 1992). The control birds expressed a large decrease in lymphocytes, thus leading to the drop in CD4 counts. An elevated CD4 count (as seen in CSF-I2 treated birds) implies increased lymphocyte reactivity as helper cells predominate, whereas a high CD8 level implies decreased lymphocyte reactivity as a result of excessive suppressor activity (Tizard, 1992). The prevention of a drop in CD4s by CSF-I2 may indicate an increased immune response, thus leading to decreased mortality.

An avian model was developed which permits assessment of the immuno-activity present in CSF-I2 within a 2 day period, thus establishing means by which the immuno-active components can be isolated and characterized. Furthermore, because the agent(s) present in CSF-I2 significantly reduced SPF mortality given a severe microbial challenge, the immuno-active components may offer even a higher degree of protection against less virulent pathogens. The mechanism by which CSF-I2 precipitates its immuno-effect is unclear. Nevertheless, our preliminary evidence indicates that one possible route by which CSF-I2 sustains an immunocompetent status is by maintaining the presence of Helper T cells in serum.

Table 3.1. Susceptibility of selected bacteria to growth inhibition by caprine serum fractionated into its high (>8 kDa) and low (<8 kDa) molecular mass (MM) components

Organism	Zone of inhibition[1]			
	Low MM		High MM	
	4 h	24 h	4 h	24 h
Gram-negative bacteria				
Pseudomonas aeruginosa ATCC 27853	0	0	0	0
Pseudomonas aeruginosa PAO1	0	0	0	0
Escherichia coli ATCC 25922	0	0	0	0
Enterobacter aerogenes	0	0	0	0
Enterobacter cloacae	0	0	0	0
Salmonella typhimurium	0	0	0	0
Pasteurella multocida ATCC 11039	IG[2]	0	IG	0
Pasteurella multocida P-1581	IG	0	IG	0
Gram-positive bacteria				
Staphylococcus aureus	0	0	0	0
Staphylococcus aureus T-5706	0	0	0	0
Bacillus subtilis	IG	0	IG	0

[1]Mueller-Hinton agar plates were streak inoculated with each of the assay organisms. Sterile filter paper disks impregnated with either the high or low molecular weight caprine serum fraction were aseptically applied to the seeded plate surfaces. The plates were incubated for 24 h at 37°C, during which time inhibition of growth in areas surrounding the disks were visually assessed at 4 and 24 h.
[2]IG = insufficient growth.

Table 3.2. A comparison of differential cell counts of control and CSF-I2 treated birds 24 h after challenge with *Pasteurella multocida* with their established baseline[1]

	Lymphocytes	Heterophils	Monocytes	Basophils	Eosinophils
baseline	69.83[a]	23.21[b]	2.53[b]	2.78[a]	1.64[a]
control	41.09[c]	46.95[a]	8.27[a]	1.43[b]	2.26[a]
treated	58.12[b]	32.52[b]	6.56[a]	1.50[ab]	1.30[a]

[a-c]Means in a column with no common superscript differ significantly ($p < 0.05$).
[1]Blood samples were taken at 24 h post-challenge. The cellular identification of 200 leukocytes was performed based on morphological characteristics as described by Lucas and Jamroz (1961), and the relative percentage of each identified cell type reported.

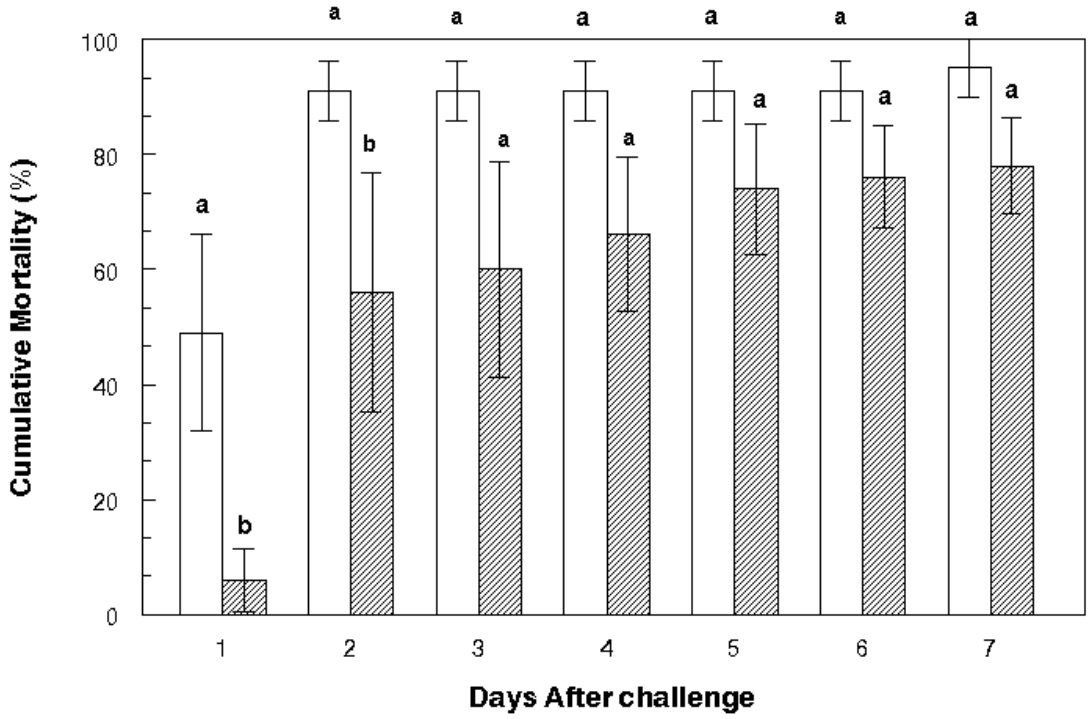

Figure 3.1. Control birds vs. treated birds (5 mg CSF-I2, days -1 and 0).

Birds were injected with 30 cfu of *Pasteurella multocida* on day 0. Control (open bar) and CSF-I2 treated (hatched bar) received either a placebo of physiological saline or 5 mg of CSF-I2 on days -1 and 0, respectively. Mortality was monitored daily for one week. Each bar represents the average, cumulative mortality per cage (n = 3) of ten birds with its associated standard experimental error. Different letters (a, b) designate differences ($p < 0.05$).

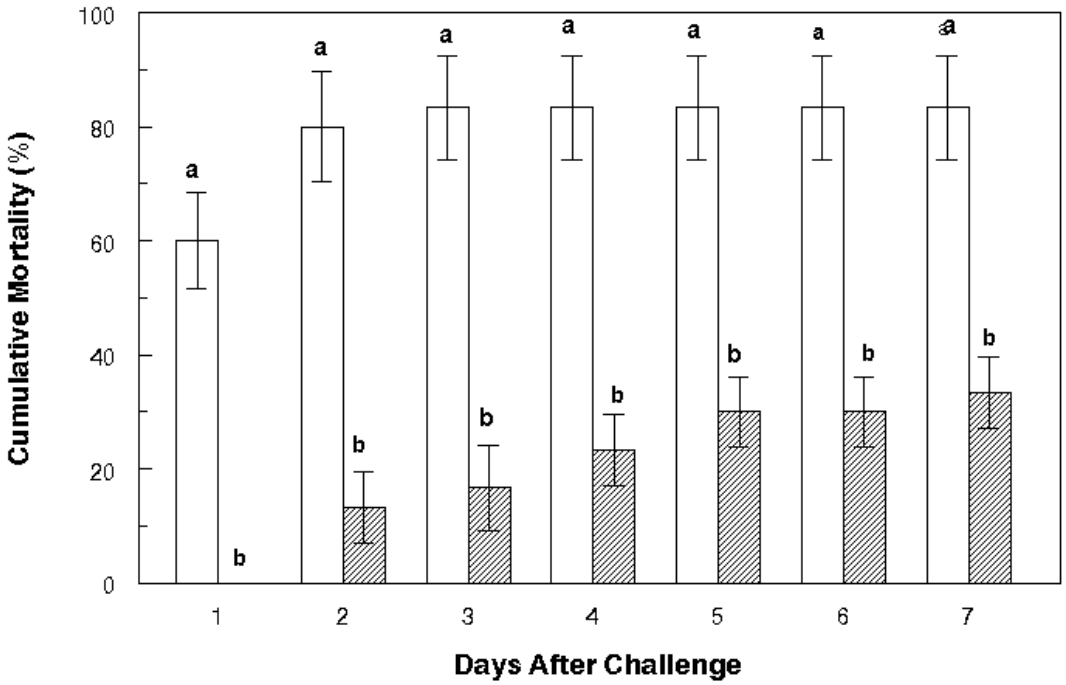

Figure 3.2. Control birds vs. treated birds (10 mg CSF-I2, days -1 and 0).

Birds were injected with 18 cfu of *Pasteurella multocida* on day 0. Control (open bar) and CSF-I2 treated (hatched bar) received either a placebo of physiological saline or 10 mg of CSF-I2 on days -1 and 0, respectively. Mortality was monitored daily for one week. Each bar represents the average, cumulative mortality per cage (n = 5) of six birds with its associated standard experimental error. Different letters (a, b) designate differences ($p < 0.05$).

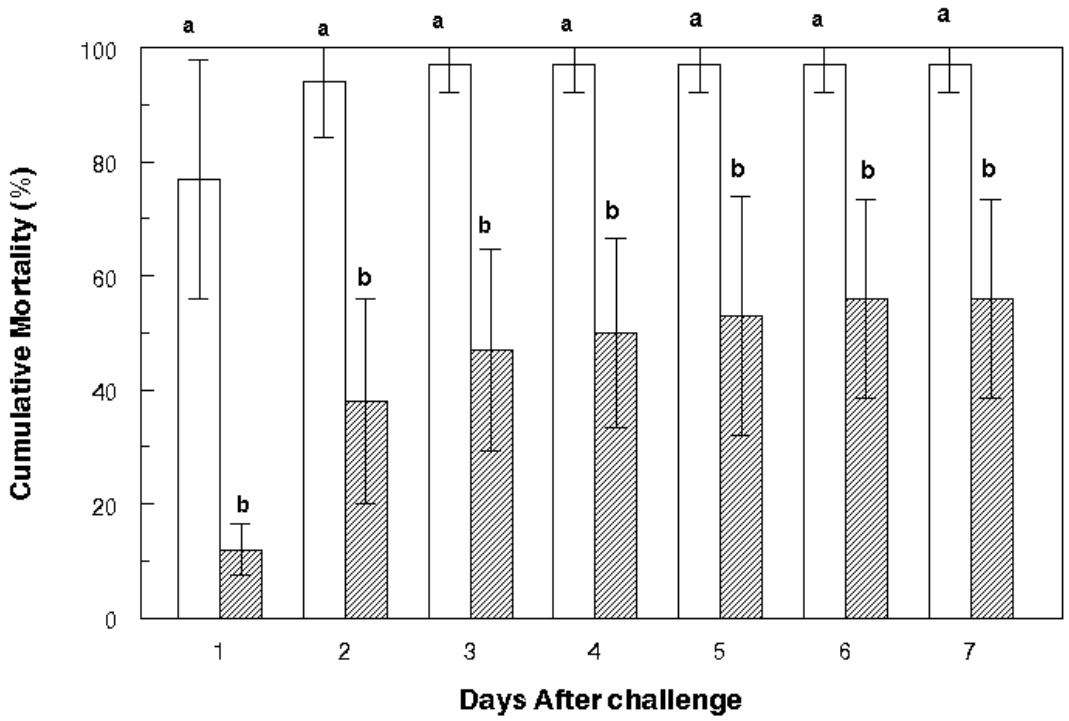

Figure 3.3. Control birds vs. treated birds (10 mg CSF-I2, days -1, 0, 1).

Birds were injected with 28 cfu of *Pasteurella multocida* on day 0. Control (open bar) and CSF-I2 treated (hatched bar) received either a placebo of physiological saline or 10 mg of CSF-I2 on days -1, 0, and 1, respectively. Mortality was monitored daily for one week. Each bar represents the average, cumulative mortality per cage (n = 5) of twelve birds with its associated standard experimental error. Different letters (a, b) designate differences ($p < 0.05$).

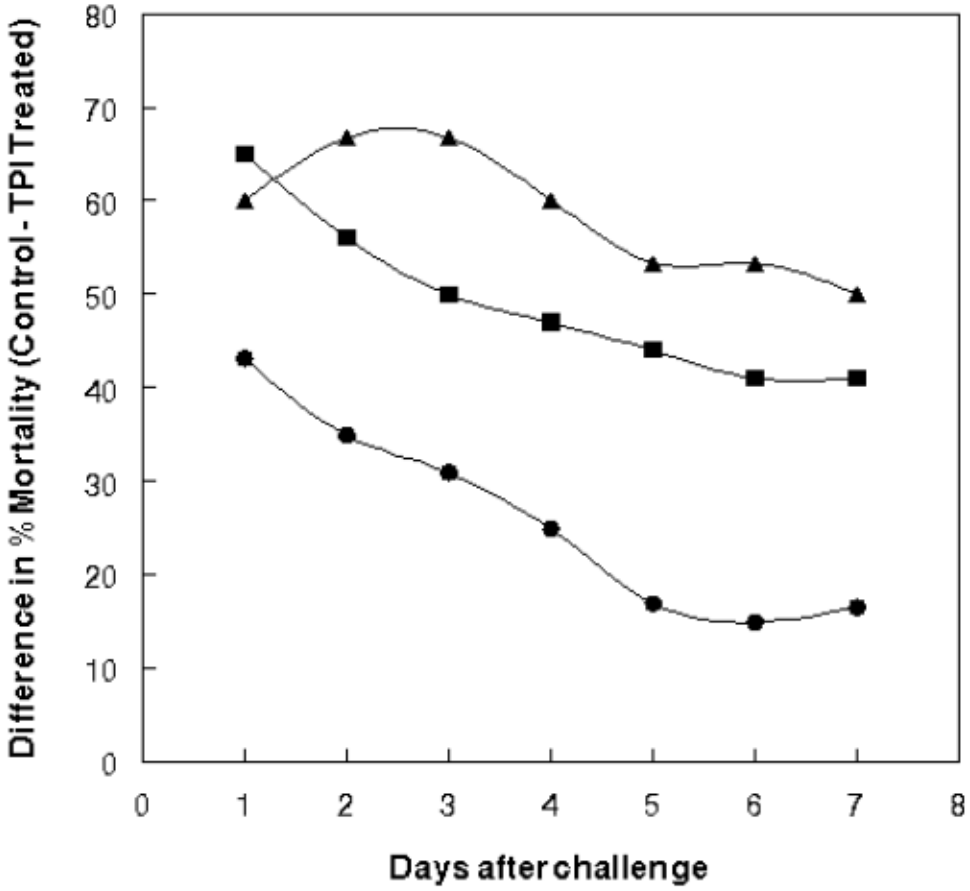

Figure 3.4. Comparison of different dosage regimens.

The percentage mortality of CSF-I2 treated birds was subtracted from its associated control group s mortality for each treatment regime: 5 mg CSF-I2 (i.m., day -1 and 0), ( ); 10 mg CSF-I2 (i.m., day -1 and 0), ( ); and 10 mg CSF-I2 (i.m., day -1, 0, and 1), ( ).

CHAPTER IV

REDUCING MORTALITY IN *SALMONELLA TYPHIMURIUM* INFECTED MICE WITH A TRI-PEPTIDIC SERUM FRACTION

INTRODUCTION

The biotechnology revolution has ushered in the age of molecular medicine and created entirely new rationale for the diagnosis, treatment, and prevention of disease. Our understanding of the fundamental biochemical processes underlying cell function grows daily. Consequently, there are burgeoning opportunities for novel strategies for the diagnosis and treatment of disease processes. In no other area of research is this more evident, and potentially more beneficial, than in the development of new medicinal agents or therapeutic regimes.

Bio-active peptides have been found to possess antiviral, antibacterial, antifungal, and wound healing properties (Sanglier *et al.*, 1993; Mizuno *et al.*, 1995; Hancock, 1999). A decameric peptide has been shown to impede the growth and spread of established tumors (Folkman, 1999). Cationic peptides help defend against the constant assault of moderate numbers of bacteria. Each natural peptide has a broad but incomplete spectrum of activity. The host compensates for this by producing an array of different peptides that together have a broader spectrum of activity, and often work in

synergy with one another. An individual may produce dozens of different peptides, and more than 500 natural cationic peptides have been described. It is believed that these defense peptides are more general in action than antibodies, and as such, have a broader range of activity (Hancock, 1999). These peptides have low toxicity to most mammalian cells and are therefore candidates for development as prophylactic agents (Maloy and Kari, 1995).

We recently fractionated caprine serum in order to isolate its complement of peptides. We partially purified a sample which when analyzed by high performance size exclusion chromatography revealed the presence of three peptides with molecular weights below 8 kD. This fraction had no bactericidal activity but when administered improved significantly the survival of chickens infected with *Pasteurella multocida* (Willeford *et al.*, 2000). This tri-peptidic isolate is identified below as CSF-I2, or Caprine serum fraction - immunomodulator 2.

In this study we develop a model to help characterize the sample s prophylactic benefit in mice. Six-week old, female Swiss Webster mice were injected intraperitoneally (i.p.) with *Salmonella typhimurium* on day 0 (~5.00 x 10^3 bacteria/mouse), effectively establishing an LD_{80} by day 7 to day 8 post-challenge. Subcutaneous injections of CSF-I2 were provided to the treated mice infected with *S. typhimurium*. Mortality was compared between mice given no CSF-I2 and those provided CSF-I2 at various periods prior to infection. Studies were performed in this manner to determine the optimal time of CSF-I2 administration and a CSF-I2 dose

response profile. Murine macrophage studies were also undertaken to determine whether administration of CSF-I2 can directly stimulate macrophage activity.

MATERIALS AND METHODS

Animal Care

Four-week old, female Swiss Webster mice were purchased from Charles River Laboratories (Wilmington, MA). The mice were acclimated for two weeks, during which time they were fed a standard maintenance diet, Laboratory Rodent Diet 5001 (PMI Feeds, Inc.), and watered *ad libitum*. Mice were group-housed (5 mice per cage) in plastic boxes bedded with wood shavings and were transferred to an isolation room immediately after inoculation with *S. typhimurium*. The isolation room was maintained at 20°C in a controlled negative pressure environment on a 12 h lighting cycle. Animal care and use were in accordance with the policies of the Institutional Animal Care and Use Committee of Mississippi State University.

Bacteria

Salmonella typhimurium (ATCC 14028) was used as the challenge organism after passaging three times through a murine host with subsequent isolation and stored in phosphate buffered saline with 10% glycerol at -80°C. This isolate was supplied from a stock culture from the Department of Biological Sciences (Mississippi State University) where it is maintained as a reference organism. Culture rehydration and cryoprotective maintenance conditions have been described previously (Darnell *et al.*, 1987).

Preparation of Caprine serum fraction - immunomodulator 2 (CSF-I2)

CSF-I2 was fractionated from goat serum by collecting the material which flowed through a dialysis membrane (Spectrum Laboratories, Inc.) with a molecular weight cut-off of 6-8 kDa. This low molecular weight material was lyophilized to a powder and stored at -70°C until reconstituted with water for use. Powdered CSF-I2 contained 37.5% protein based upon nitrogen content.

Experimental Design

Mice comprising the control and treated populations were injected i.p. with 0.1 ml of *Salmonella typhimurium* (~5.00 x 10^3 bacteria/mouse) on day 0. Unless stated otherwise, treated mice were given a 0.25 ml subcutaneous injection of CSF-I2 (5 mg) at the time designated by the experimental protocol, while control mice received a placebo of physiological saline. Negative control mice were sham handled in a similar manner to the control and treated populations to evaluate the influence of non-experimental parameters on mortality. To obtain statistical significance, mice were housed five per cage and a minimum of five cages were used per treatment group. Mice were monitored three times daily and mortality recorded until 80% of the control mice died.

Effect of Heat Treatment on CSF-I2

CSF-I2 (20 mg/ml) was brought to 85°C for 30 min and 5 mg doses were administered to mice according to the experimental design described above.

Limbulus amebocyte Lysate (LAL) Endotoxin Quantitation Assay

The standardized endotoxic activity for two independent preparations of CSF-I2 was determined by Associates of Cape Cod (Woods Hole, MA) using an LAL gel clot assay. The threshold sensitivity of the reaction was 0.03 endotoxic unit (EU) per ml.

Cell Culture

RAW 264.7 cells (murine alveolar macrophage line) were obtained from American Tissue Culture Collection (ATCC TIB-71, Manassas, VA) . Cells were cultured in a complete medium [Dulbecco s Modified Eagle s Medium (DMEM) adjusted to contain 4.5 g/L glucose, 1 mM sodium pyruvate, 1.5 g/L sodium bicarbonate, 4mM L-glutamine, 100 units/ml penicillin, 100 g/ml streptomycin, and 0.25 g/ml amphotericin with a final concentration of 10% fetal bovine serum] specified by ATCC. Cells were maintained at 37ºC in a 5% CO_2 humidified incubator.

Nitrite Quantitation

A Griess Reagent Kit (Molecular Probes, Eugene, OR) was used to determine nitrite (NO_2) levels produced by stimulated and non-stimulated macrophages. Cells were plated at 1 x 10^5 cells/cm^2 in 24-well culture plates and allowed to adhere for four hours. Medium was then removed and replaced with complete medium (control wells), or complete medium supplemented with CSF-I2 (0.1, 1, or 10 mg/ml) or 50 g/ml lipopolysaccharide (LPS serotype O127:B8; Sigma, St. Louis, MO) for 72 h (n=4). Separate plates were used for the three time points (i.e., 24, 48, and 72 h). Supernatant

(150 l) was removed at the designated time and mixed with 20 l Griess reagent (0.1% naphthylethylene diamine dihydrochloride, 1% sulfanilamide, 3% H_3PO_4) and 130 l ddH_2O in a 96 well tissue culture plate. The microplate was then incubated at 37°C for 30 min and absorbance read at 548 nm on a Quant Plate Reader (Biotek Instruments, Winooski, VT), with a standard curve generated with sodium nitrite.

Protein Determination

Protein determinations of adherent macrophages were made in order to standardize nitrite production. Supernatant was removed for nitrite analysis and the well was washed with sterile PBS. Adherent cells were lysed by adding 1 ml 0.3M NaOH and incubating for 30 min at room temperature. A Bradford assay for microplates (Bio-Rad, Hercules, CA) was run in triplicate as per kit instructions. Briefly, 20 l of the lysis supernatant and 200 l Bradford reagent were added to a well and incubated at room temperature for 30 min. Absorbance was then measured on a Quant Plate Reader at 595 nm. A standard curve was prepared from bovine serum albumin (BSA) in 0.3M NaOH.

Statistical Analysis

All experiments were arranged in a completely randomized design. Data demonstrating cumulative mortality were analyzed separately for each time point using one way analysis of variance (ANOVA) with the general linear models procedure (PROC

GLM) and the means separated by Fisher s protected LSD procedure (SAS Institute Inc., 1997). A p value less than 0.05 was necessary to be considered significant.

RESULTS

LAL Endotoxin Quantitation Assay

Two separate preparations of CSF-I2 were analyzed for endotoxic activity. Each preparation had equivalent endotoxin levels of 0.8 EU/mg.

Effect of Various Treatment Regimens on the Resistance of Mice to S. typhimurium

The effect of varying the timing, dosage, and frequency of CSF-I2 pretreatment on the survival of mice to a potentially lethal challenge with *S. typhimurium* was investigated in order to determine the optimal administration for CSF-I2. A single dose of CSF-I2 was administered at various times prior to challenge to assess persistence of its positive effects and to ascertain the optimal time of its administration with regard to the *S. typhimurium* challenge model. CSF-I2 was administered on either day -4, -2, -1, or coincident with the challenge on day 0 (Figure 4.1). Four days were usually required before deaths were observed in control populations of female Swiss Webster mice challenged with *S. typhimurium* (~5 x 10^3 bacteria/mouse). A rapid rise in death ensued with approximately 80% mortality occurring 8 days post-challenge. Mice treated with CSF-I2 four days prior to challenge showed no significant difference from the control. Prophylactic benefit, however, was observed if CSF-I2 was given on either day -2 or 0. By day 8 the control population reached 80% mortality, while groups that received

CSF-I2 on either day -2 or 0 had percent mortalities of 60 and 54, respectively. Mice treated one day prior to challenge had the least number of deaths. Only 32% of this treated group was dead by 8 days post-challenge. Beginning with day 5 post-challenge (when mortality was established in this control population) there was a statistically significant difference between the control group and each of the day -2, -1, and 0 CSF-I2 treated groups. The day -1 treatment group also had significantly lower mortality than the day 0 and day -2 CSF-I2 populations as exemplified by the day 8 p values of $p = 0.0193$ and $p = 0.0014$, respectively.

A dose response study was performed in order to determine the optimal amount of CSF-I2 to administer for prevention of mortality (Figure 4.2). CSF-I2 was prepared so that a subcutaneous injection would deliver either 0.1, 5, 15, or 20 mg CSF-I2. All injections of CSF-I2 were given on day -1, as this was shown by the time course study to produce the greatest prophylactic benefit. On day 7, all doses were significantly different ($p < 0.05$) from the control mice except for the 0.1 mg CSF-I2 dosage. Eighty three percent of the control population died by day 7, while with respect to increasing amounts of CSF-I2, 73.3, 33.3, 13.3, and 13.3% had died in these treated groups.

Onset of mortality typically occurred 4 days post-challenge. It was of interest to determine whether survival could be improved with supplemental administrations of CSF-I2. Control mice were compared to treated groups which received CSF-I2 on day -1, on days -1 and 1, and on days -1 and 2 (Figure 4.3). All treated groups had significantly fewer deaths than the control population between days 5 and 8. There was

no significant difference in prophylactic benefit however between the populations which received either a single or multiple administrations of CSF-I2.

Effect of Heat Treatment on CSF-I2

When CSF-I2 was incubated at 85°C for 30 min the material no longer provided prophylactic benefit. The mortality profile presented in the murine population treated with heated CSF-I2 (5 mg) was indistinguishable from the control population.

Macrophage Nitrite Production

The macrophages were stimulated by LPS to produce a significant increase in nitrite production over the control cells by day 2 ($p < 0.0001$). CSF-I2 did not stimulate macrophage activity (as detected by nitrite production) when tested up to a concentration of 10 mg/ml (Figure 4.4). Neither cytotoxic nor proliferative effects were observed due to the administration of CSF-I2.

DISCUSSION

Agents which retard pathogenesis may enable a host to mount a successful defense to challenges of the immune system. These agents can provide specific protection (i.e., in the form of antibodies) or be general in nature and enhance overall immunocompetence. Cytokines and cationic peptides are two such classes of non-specific defense agents. We were able to isolate a mixture of three peptides which have an estimated molecular mass of approximately 5 kDa, as determined by size exclusion

chromatography (data not shown), from caprine serum which when administered to mice challenged with *S. typhimurium* significantly reduced ensuing mortality. The prophylactic benefit was issued in a dose dependent manner with a maximal effect garnered when approximately 15 mg CSF-I2 was administered. Benefit appears to derive from CSF-I2's proteinaceous components in light of the observation that all benefit is lost upon incubation at 85°C, a procedure known to denature protein. Support for this conclusion was derived from the observation that when CSF-I2 was digested overnight with the proteases Bromelain and Proteinase K, loss of therapeutic benefit was observed.

CSF-I2 showed no bactericidal activity when surveyed against a battery of both gram-positive and gram-negative bacteria (Willeford *et al.*, 2000) and did not contain a level of endotoxin sufficient to promote a pyrogenic response. However, its administration is known to retard bacterial pathogenesis in chickens (Willeford *et al.*, 2000) and mice with the greatest survival observed when given approximately 24 hr before challenge. Significant benefit was observed when CSF-I2 was given at the time of challenge or two days before challenge but no benefit was observed when administered four days before a severe challenge.

Clonal and non-clonal immunity can be found in serum in the form of immunoglobins, collectins, cytokines, chemokines, and cationic peptides (Janeway and Travers, 1997). Isolation of CSF-I2 effectively excludes globulins (180 kDa), collectins (a collection of multimeric proteins with a subunit molecular mass of approximately 50 kDa), and cytokines (~10-80 kDa) (Tizard, 1996; Nair *et al.*, 2000). Chemokines (~8 kDa) function mainly as chemoattractants for phagocytic cells,

recruiting monocytes and neutrophils from the blood to sites of infection (Janeway and Travers, 1997). Molecular mass estimates point more favorably towards a cationic peptide, some of which have a mass as low as 2 kDa. Cationic peptides have been found to have diverse physiological roles *in vivo* (Sanglier *et al.*, 1993; Mizuno *et al.*, 1995; Hancock, 1999), but are primarily associated with bactericidal activity with an MIC in the range of 1-100 g/ml (Martin *et al.*, 1995; Hancock, 1997). CSF-I2 at concentrations over 2,500 times the upper MIC range recognized for cationic peptides produced no bactericidal activity against *Salmonella typhimurium* (Willeford *et al.*, 2000). Preliminary sequence data shows, however, a significant presence of lysine and arginine, which is consistent with the nature of cationic peptides. The bio-active peptide(s) in CSF-I2 may initiate an atypical or unclassified response for a cationic peptide.

It is unclear whether administration of CSF-I2 affects the ability of the host to kill the bacteria, control their subsequent rate of growth, or both. However, CSF-I2 influences in a positive way the ability of the host to withstand and survive a challenge of an infectious agent.

Studies are being pursued in order to elucidate CSF-I2's mechanism of action. While CSF-I2 does not appear to work through directly stimulating macrophages, it remains possible that CSF-I2 is causing an indirect stimulation through the production of macrophage activating effector molecules such as IFN or TNF.

The search for agents which potentiate the immune response is a driving force in drug research. Use of immune regulating or immune modulating molecules should induce a state of general hyperactivity of the immune system which should precipitate

and/or enhance the prophylactic response to immune challenges such as pathogenic infection. If such a non-specific immune response could be initiated at will, it could be utilized to either act alone or in conjunction with conventional treatments directed towards etiological agents and the progression of a diseased state. CSF-I2 appears to possess an immunomodulatory activity. Further investigation is required to determine the nature of the immune-regulatory compound and elucidate its mode of action.

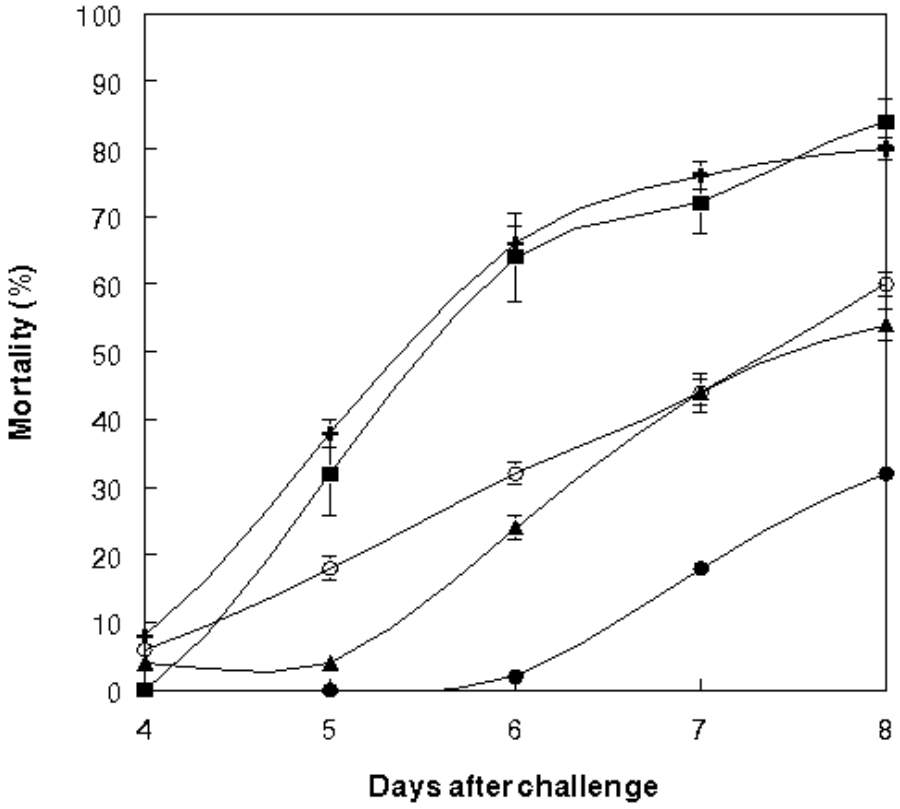

Figure 4.1. Optimal Time of CSF-I2 Administration (Time Course Profile).

Control mice were administered 0.1 ml (~5 x 10^3 cfu) of *S. typhimurium* i.p. on day 0 (+), while treated mice received both *S. typhimurium* on day 0 and a 0.25 ml subcutaneous injection of CSF-I2 (5 mg) either on day -4 ( ), day -2 ( ), day -1 ( ), day 0 ( ). Each data point represents the average daily mortality (n = 5) with its associated SEM per cage of 5 mice.

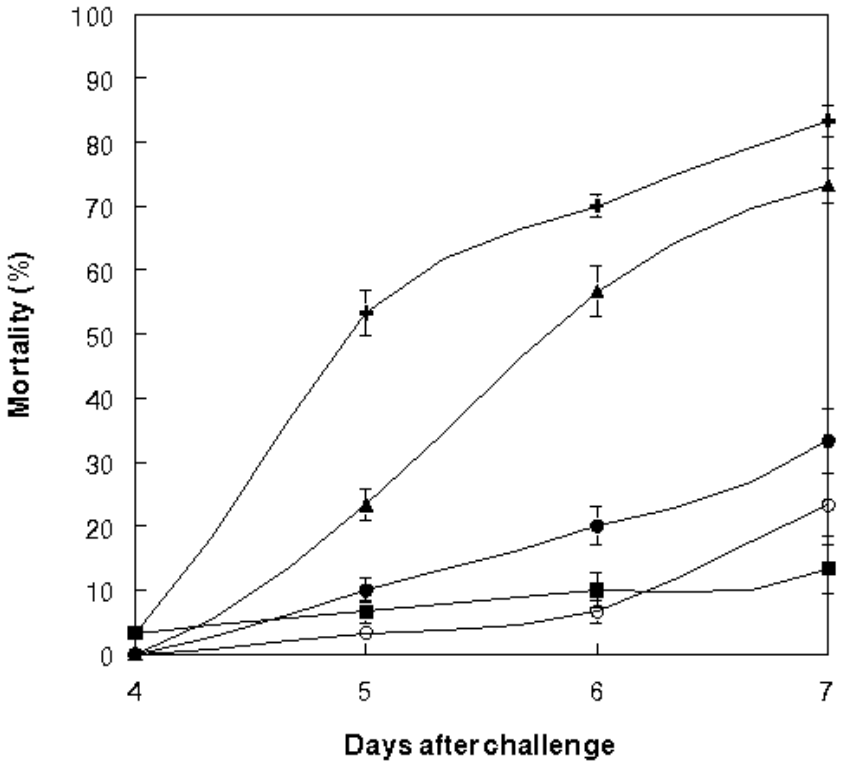

Figure 4.2. CSF-I2 Dose Response Profile.

Control mice were administered 0.1 ml (~5 x 10^3 cfu) of *S. typhimurium* i.p. on day 0 (+), while treated mice received both *S. typhimurium* on day 0 and a subcutaneous injection of either 20.0 mg ( ), 15.0 mg ( ), 5.0 mg ( ), or 0.1 mg ( ) CSF-I2. Each data point represents the average daily mortality (n = 6) with its associated SEM per cage of 5 mice.

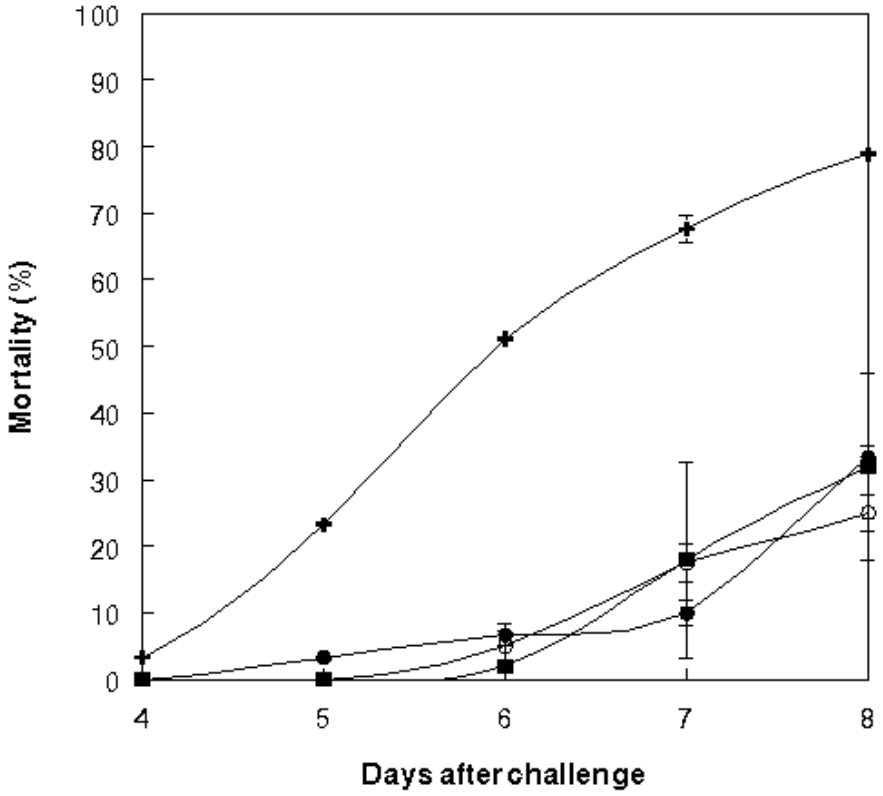

Figure 4.3. Effect of Multiple Administrations of CSF-I2.

Control mice were administered 0.1 ml (~5 x 10^3 cfu) of *S. typhimurium* i.p. on day 0 (+), while treated mice received both *S. typhimurium* on day 0 and a 0.25 ml subcutaneous injection of CSF-I2 (5 mg) on either day -1 ( ), days -1 and 1 ( ), or on days -1 and 2 ( ). Each data point represents the average daily mortality (n = 6) with its associated SEM per cage of 5 mice.

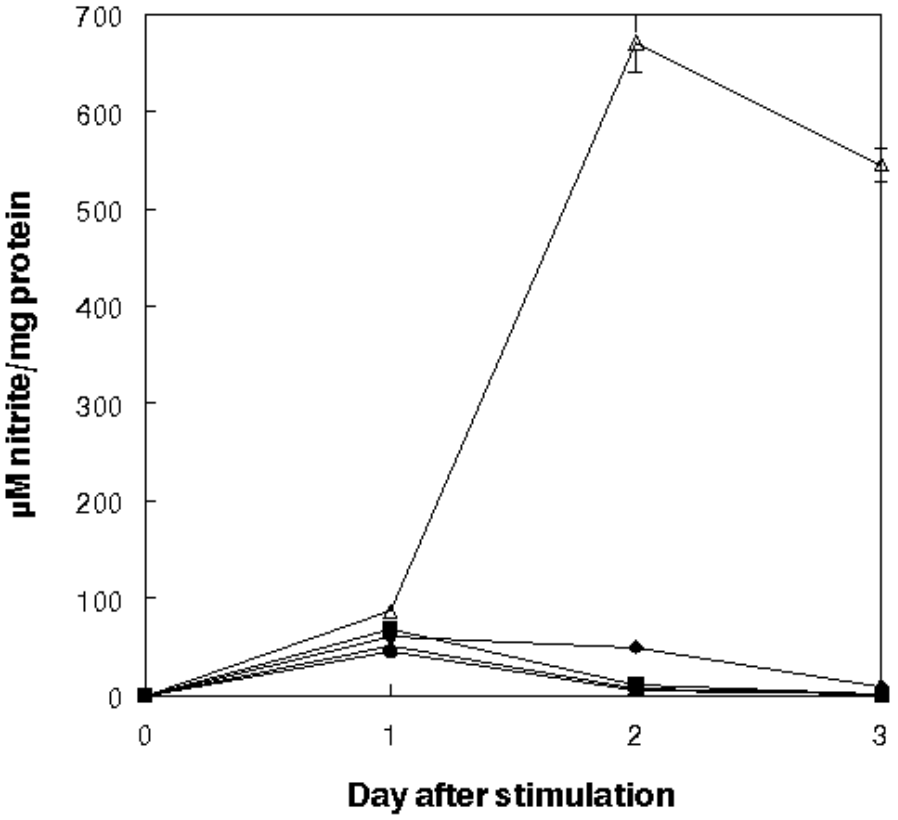

Figure 4.4. Macrophage nitrite determination.

Macrophages were plated at 1 x 10^5 cells/ml in 24 well tissue culture plates. After four hours, medium was removed and replaced with complete medium ( ) or complete medium supplemented with CSF-I2 (0.1, ; 1, ; and 10 mg/ml,) or 50 g/ml LPS ( ). Nitrite and protein were assessed at 24, 48, and 72 h. The results are expressed as the mean M nitrite produced/mg adherent protein with its associated SEM from four wells.

CHAPTER V

IMPROVED SURVIVAL OF CANINES WITH PARVOVIRUS WHEN TREATED WITH A TRI-PEPTIDIC SERUM FRACTION

INTRODUCTION

Canine parvovirus (CPV) was first identified in Greece in 1974 and by 1978 had spread throughout Europe and the United States. Cases were diagnosed worldwide by 1979 (Pollock and Coyne, 1993). Infection is acquired by ingestion of infective virus and may spread quickly from dog to dog. The virus requires rapidly dividing cells for its replication (Dunn *et al.*, 1995a), thus targeting the intestinal tract, lymphoid tissues, and bone marrow, with marked plasma viremia observed three to five days after infection and enteric distress shortly thereafter (Pollock and Coyne, 1993). Dogs from 1-6 months of age should be considered at risk of CPV infection, but the most severe, often fatal, incidence of CPV occurs in puppies less than three months of age (Tilley and Smith, 1997).

CPV is a small, non-enveloped single-stranded DNA-containing virus (Parrish, 1999) that is genetically and antigenically closely related to two other parvoviruses, feline panleukopenia virus and mink enteritis virus (Parrish *et al.*, 1982). Vaccines have been developed which provide long-term, if not complete, immunity. However, such

protective measures are effective only if given when maternally acquired CPV antibodies have declined to the point where the puppy has become susceptible to infection, and thus responsive to vaccination. This period typically extends from 4-16 weeks of age. If the disease is acquired during this period of susceptibility, a key determinant in the animal s survival depends on its ability to limit the magnitude and duration of viremia. Dogs which survive the acute stage of infection usually recover fully and possess lasting immunity (Pollock and Coyne, 1993).

Supportive care, the cornerstone of CPV therapy, provides time for the host to mount an effective immune response. Supportive therapy consists mainly of fluid replacement, restoration of acid/base balance, antiemetics, antibiotic treatment to prevent secondary bacterial infection, and restrictive diet (Pollock and Coyne, 1993; Dunn *et al.*, 1995b). Other than supportive therapy, only recombinant feline interferon- - INTERCAT (Toray Industries, Tokyo, Japan) has been shown to be effective for CPV infection. It s mode of action is thought to derive primarily from interferon s antiviral activity, but INTERCAT may also activate immunological responses (Minagawa *et al.*, 1999).

Discovering agents which potentiate the immune response is a driving force in drug research. Cytokines and cationic peptides are two classes of compounds found in serum which have shown promise in this area of research. Bio-active peptides have been observed to inhibit the growth and spread of established tumors, promote antiviral, antibacterial, antifungal, and even wound healing properties (Sanglier *et al.*, 1993; Mizuno *et al.*, 1995; Folkman, 1999; Hancock, 1999). It is believed that these defense

peptides are more general in their actions than antibodies, and as such, have a broader range of activity (Hancock, 1999).

Caprine serum fraction - immunomodulator 2 (CSF-I2) was isolated from serum and clinically evaluated for therapeutic benefit. CSF-I2 had no bactericidal activity, but when administered improved the survival of chickens infected with *Pasteurella multocida* (Willeford *et al.*, 2000) and mice infected with *Salmonella typhimurium*. There have been no studies, however, to determine whether CSF-I2 provides therapeutic benefit against a viral challenge. The purpose of this study was to determine the efficacy of CSF-I2 as a supplement to conventional supportive therapy for CPV infection.

MATERIALS AND METHODS

Selection Criteria

Animals between eight and twelve weeks of age were included in the study if diagnosed with canine parvovirus by fecal ELISA examination. Animals were excluded from the study if they were unconscious or died within 8 h of admission.

Supportive Therapy

Treatment at the veterinary clinic (St. Paul, MN) depended upon the animal s presenting condition and included fluid replacement, antiemetics, anti-endotoxic agents, and/or antibiotics for treatment of possible secondary infections.

Experimental Design

All dogs diagnosed for CPV through a fecal ELISA examination (IDEXX, Westbrook, ME) were immediately placed on supportive therapy. Upon owner consent, a subpopulation of this group was administered CSF-I2 therapy (Willeford *et al.*, 2000). This consisted of a single intramuscular (i.m.) injection of 0.5 ml CSF-I2 (3 mg/ml). After three days, the dogs were given a second injection of CSF-I2 and assessed for their response to initial therapy. An additional follow-up was conducted one week later.

Cell Culture and Medium

Canine fibroblast A-72 cells from the American Type Culture Collection (ATCC CRL-1542) were cultured in Eagles minimal essential medium supplemented with 5-10% (v/v) heat inactivated Fetal Bovine Serum, 100 units/ml penicillin, 10 g/ml gentamicin, and 2.5 g/ml fungizone. Cultures were maintained as monolayers in tissue culture labware at 36-38ºC in a humidified atmosphere of 5-7% CO_2.

Canine Parvovirus

Canine parvovirus (VR-2017) was originally obtained from the ATCC and cryogenically stored at -70ºC. The virus was propagated in A-72 cells in disposable tissue culture labware as described above.

Virucidal/Antiviral assays

The virucidal and antiviral interactions between CSF-I2 and CPV were assessed by ViroMed Biosafety Laboratories (St. Paul, MN). In the virucidal assay, CPV was incubated with dilutions of 100 - 0.001 g/ml CSF-I2 for four hours, and then added to wells containing canine fibroblast A-72 cells (test wells). Wells were also established to represent a cell control (cells which are neither exposed to CPV or CSF-I2) and virus control (cells which are exposed to CPV following incubation with culture medium). After incubation of the cells for 12 days, the supernatants from the cell control, virus control and CSF-I2 treated wells were assayed for virucidal activity using a hemagglutination assay. In order to determine whether CSF-I2 was directly cytotoxic to the canine fibroblasts, cells were plated and exposed to CSF-I2. After incubating for 12 days, the wells were assessed for cellular proliferation using a Microculture Tetrazolium Assay (Delhaes *et al.*, 1999).

In the antiviral assay, canine fibroblast A-72 cells were exposed to CPV for 2 h and then either CSF-I2 (up to 100 g/ml) or culture medium was added to establish the treated and virus control samples. Wells plated with cells that had neither been exposed to CPV nor CSF-I2 were used to establish a cell control well. Cells were incubated for 10 days and their supernatants assayed for viral infectivity using a hemagglutination assay.

RESULTS AND DISCUSSION

A field study incorporating data from 33 clinics in Japan showed that canines which received supportive therapy for CPV (n = 21) experienced a near 60% mortality (Minagawa *et al.*, 1999). All data used in our study were obtained from a veterinary clinic located in St. Paul. MN. When canines were diagnosed with CPV and administered supportive therapy at this site, the mortality was 45% (n = 150).

A total of 74 CPV cases diagnosed at this center also received supplemental CSF-I2 therapy. Fifty-six dogs, confirmed to have contracted CPV based upon fecal ELISA examination, were placed immediately on supportive therapy and administered CSF-I2. Six deaths were reported, resulting in a mortality of 10.7% (6/56), well below the average for dogs treated solely with supportive therapy at this site and that reported by Minagawa *et al.* (1999). Fourteen dogs were diagnosed with CPV based upon their presentation of clinical symptoms. With supportive therapy supplemented by CSF-I2 there was only one recorded death. Littermates to CPV patients may test negative for CPV and then abruptly show CPV symptomology. Four CPV littermates were treated with CSF-I2, and no deaths were recorded. Of the 74 cumulative CPV cases where conventional treatment was supplemented by administration of CSF-I2, there were 7 deaths; constituting a collective mortality of 9.5% (Fig. 5.1). When treatment incorporated the use of CSF-I2, patients received substantial benefit, as evidenced by their increased survival.

INTERCAT, an approved veterinary antiviral agent, has been shown to improve upon conventional CPV therapy. A total of 93 CPV dogs from thirty-three veterinary

clinics in Japan were administered INTERCAT once a day for 3 consecutive days at a dose of 1 or 2.5 million units/kg. Mortality dropped from 61.9% (13/21) in the control group to 19.4% (14/72) when INTERCAT was utilized (Minagawa *et al.*, 1999). It is clear that conventional CPV therapy can be improved upon, as both CSF-I2 and INTERCAT were able to lower the incidence of death by approximately forty percent. It would be of interest to evaluate whether these adjunct therapies are able to act in concert to further improve survivability.

CSF-I2 may also impart antiviral activity. Therefore it was necessary to determine whether CSF-I2 has the ability to directly kill CPV (i.e., possesses *in vitro* virucidal activity). In order to assess CSF-I2's virucidal activity to CPV it was necessary to first establish whether the viability of the A-72 host cell line was impaired by the presence of CSF-I2. Cell proliferation was not affected adversely when exposed to CSF-I2 at concentrations from 0.001 - 100 g/ml CSF-I2 (Figure 5.2). CSF-I2 was not virucidal to CPV as infectivity and proliferation were not affected by prior exposure to CSF-I2.

The *in vivo* antiviral activity of CSF-I2 was also assessed in this cell line. There was no evidence that CSF-I2 promoted antiviral activity within the host cell. While canine fibroblast A-72 cells are used to support growth of CPV, they were not developed to be immunocompetent. Isolated cell lines often lack the ability to mount a full immune response.

It is clear from the data provided that CSF-I2 promotes resistance to CPV infection without acting as a virucidal agent. The mechanism by which it accomplishes

this task remains to be elucidated. It has also been reported that while CSF-I2 has no bactericidal activity it enables a recipient to better withstand a potentially lethal bacterial challenge (Willeford *et al.*, 2000). CSF-I2 appears to provide therapeutic benefits against both microbial and viral challenges. Future studies are required to determine the full extent of CSF-I2 s bioactivity and mode of action.

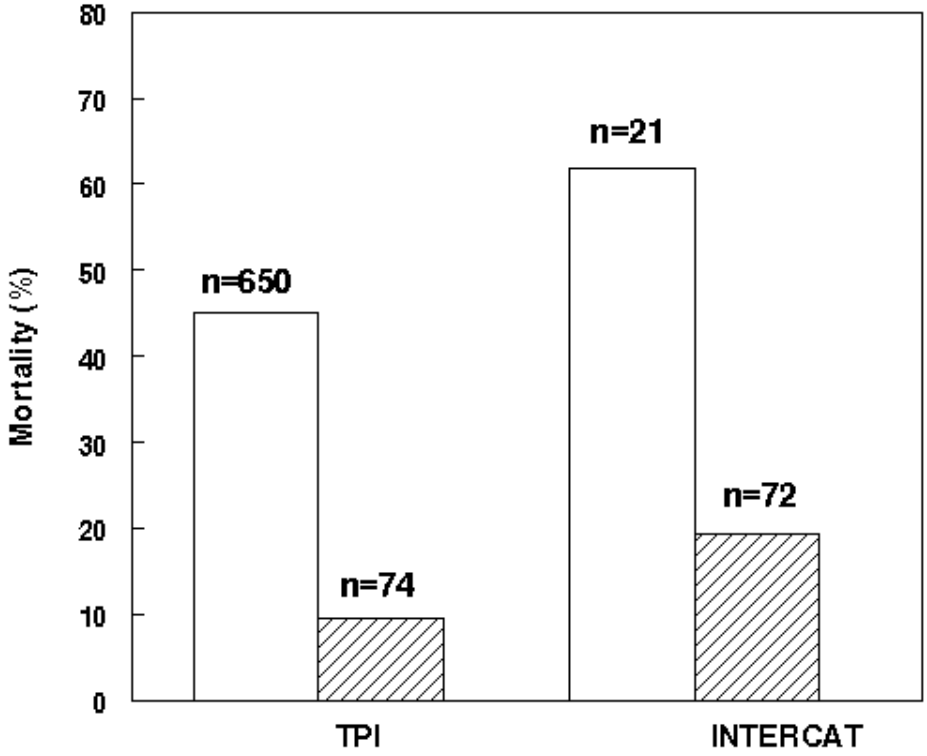

Figure 5.1. Improving conventional CPV therapy by administration of CSF-I2 or the antiviral agent INTERCAT.

The open bar represents the mortality present in the control group of dogs which were treated for CPV by supportive therapy while the hatched bar shows the mortality when conventional therapy was supplemented by administration of CSF-I2 (see Materials and Methods for details) or INTERCAT (Minagawa *et al.*, 1999).

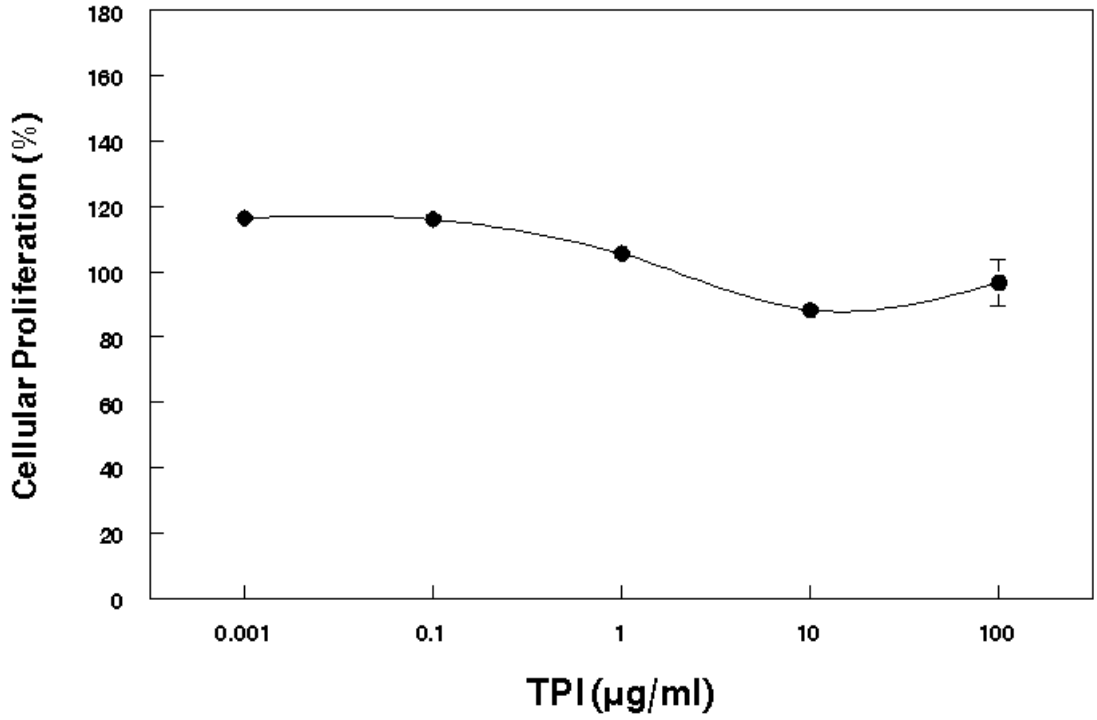

Figure 5.2. Cytotoxicity Assay.

Canine fibroblast A-72 cells were plated and exposed to dilutions of CSF-I2 from 100 - 0.001 g/ml for four hours. The cells were then incubated for 12 days, and a Microculture Tetrazolium Assay was used to assess cellular proliferation. Each point represents the average of the replications (n=3) for each concentration of CSF-I2 tested relative to the average proliferation of cells not exposed to CSF-I2 with its associated standard experimental error.

CHAPTER VI

ISOLATION OF AN IMMUNOMODULATORY CAPRINE FACTOR

INTRODUCTION

Studies conducted in our laboratory revealed the presence of a goat serum factor that significantly improved the overall survival of animals experiencing potentially lethal pathogenic challenges (Willeford *et al.*, 2000; Willeford *et al.*, 2001; Hamm *et al.*, 2002). The physical nature of this factor was unknown; the size, composition and mode of action were not elucidated.

The crude goat serum isolate was found to possess carbohydrates, lipids and proteinaceous material. All three of these broad classes of biological compounds have been observed to produce functional immunomodulators (e.g., acemannan, lipid monophosphoryl A, and cationic peptides) (Hadden, 1993; Abbas and Janeway, 2000; Hamm *et al.*, 2002). Before a purification protocol could be designed, it was important to establish a reliable assay and identify which class of biological compounds generated the biological activity of interest. To date, the only proven assay denoting the factor s bioactivity is derived from animal model studies and should be considered therefore relatively insensitive.

Upon conducting a thermal stability study it was observed that biological activity was lost when material was incubated at 85ºC for 30 minutes or 100ºC for 15 minutes. This result indicated that the factor was proteinaceous in nature. This hypothesis was verified with the loss of bioactivity after proteolytic digestion.

All subsequent studies involving purification centered around the hypothesis that the active agent(s) were proteinaceous. Moreover, caution had to be exercised also to retain immunomodulatory activity, as it was the only marker for the factor s presence. Purification procedures were employed which permitted initially the large scale or bulk separation of serum components (e.g., proteins versus peptides). Analytical resolution was subsequently used to characterize the nature of the bulk-isolated material (e.g., molecular weight and amino acid analysis). Studies reported here reflect the progress of this effort.

MATERIALS AND METHODS

Protein Denaturation Studies

Effluent obtained after equilibrium dialysis (6-8 kDa MWCO) was performed was concentrated to 20 mg/ml and either digested overnight with 0.36 units/ml bromelain and 2 mg/ml proteinase K at 4ºC or incubated at either 85ºC for 30 min or 100ºC for 15 min. After these treatments were performed, the bioactivity was assessed according to the *Salmonella typhimurium* model previously described.

Equilibrium Dialysis

Molecular porous membrane tubing (Spectrum Laboratories, Inc) with a molecular weight cut off (MWCO) range of 12-14 kDa, 6-8 kDa, or 1 kDa was used to perform equilibrium dialysis. Caprine serum (Colorado Serum Co.) was dialysed at 4ºC against approximately ten volumes of double distilled deionized water for 24 hours. Material which flowed through the dialysis membrane (caprine serum fraction - immunomodulator 2 or CSF-I2) was lyophilized (Lyph Lock 6, Labconco, Kansas City, MO) to a powder and stored at -70ºC until reconstituted with water for use.

Centrifugal Ultrafiltration

Ultrafiltration was conducted on CSF-I2 by using a centrifugal force of 5000 x g to drive material through an Amicon Centricon YM-30 (30 kDa MWCO), YM-10 (10 kDa MWCO) or YM-3 (3 kDa MWCO) in accordance to manufacturer s instructions. If the sample volume was greater than 10 ml an Amicon Centriprep YM-10 (Millipore Corp, MA) apparatus operated at 3000 x g was used.

Size Exclusion Chromatography

Size exclusion chromatography was performed with either a Superdex 200 HR 10/30 (Pharmacia LKB Biotechnology, Uppsala, Sweden) or a TSK G2000 SWXL (Phenomenex, Torrance, CA, 300mm x 7.8mm).

Twenty l aliquots of sample (i.e, caprine serum, CSF-I2, or TPI) were applied to a Superdex 200 HR 10/30 size exclusion column equilibrated in 10 mM sodium

phosphate (pH 7.1) and 150 mM NaCl. The column was calibrated with BioRad gel filtration chromatography standards [thyroglobulin (670 kDa), IgG (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and Vitamin B_{12} (1.35 kDa)]. The operating system used was a Hewlett Packard 1100 HPLC operated at a flow rate of 1 ml/min and set to detect at 280 nm.

Twenty-five l of TPI (10 mg/ml) or a mixture of reference proteins was applied to an analytical TSK G2000 SWXL size exclusion column equilibrated with 200mM potassium phosphate (pH 6.8) containing 15mM NaCl. The column was calibrated with BioRad gel filtration chromatography standards. The system was operated at 0.5 ml/min, the eluate was monitored at 214nm, and 0.5 ml fractions were collected. This procedure was conducted by Commonwealth Biotechnologies, Inc. (Richmond, VA).

SDS Gel Electrophoresis

Electrophoresis in the presence of sodium dodecyl sulfate was performed according to the discontinuous system developed by Laemmli (1970) with a 4% acrylamide stacking gel (pH 6.8) and a 15% acrylamide resolving gel (pH 8.8) utilizing a BioRad Mini-PROTEAN II electrophoresis unit. Low MW standards obtained from BioRad [i.e., phosphorylase b (97.4 kDa), serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), and aprotinin (6.5 kDa)] were used as a molecular weight reference guide. Proteins were visualized utilizing a BioRad silver stain (Hercules, CA) as per kit instructions.

Reverse Phase HPLC

Reverse phase HPLC was performed with a C-18 HPLC column (Alltech Absorbosphere C-18 5U, 150mm x 4.6mm) equilibrated in 0.1% aqueous TFA (trifluoroacetic acid). The column (attached to a Waters 600 E HPLC) was washed with this buffer for 10 min (1 ml/min) and bound material eluted by establishing a binary gradient which linearly increased the secondary buffer (80% acetonitrile, 0.1% aqueous TFA) to 100% over a 20 min period. A 100 l aliquot of TPI in 0.1% TFA was applied to the column. Sample elution was monitored at 220 nm with an Agilent 1100 Series diode array detector, and peak fractions were spectrophotometrically scanned from 200-400nm.

Amino Acid Analysis

Aliqouts obtained from the peak fractions resolved by TSK G2000 SWXL size exclusion chromatography were dried and hydrolyzed by gas phase HCl for 20 h at 110ºC. Following hydrolysis, the samples were dried, suspended in 0.4M borate (pH 10.2) and derivatized. Primary amino acids were derivatized with ortho-phthaladehyde and secondary amino acids were derivatized with 9-fluorenylmethoxy-carbonyl. Derivatives were separated by reverse phase HPLC and detected by UV absorbance. Quantitation was done with the Aminoquant software (Agilent Technologies, Palo Alto, CA) calibrated with Agilent standards. This procedure was conducted by Commonwealth Biotechnologies, Inc. (Richmond, VA).

RESULTS

Size exclusion methodologies were used to fractionate caprine serum. Caprine serum was initially separated into large and small molecular weight components by subjecting it to equilibrium dialysis. The small molecular weight material isolated when dialysis was conducted with molecular porous tubing possessing a MWCO of 12-14 kDa or 6-8 kDa (CSF-I2) was able to inhibit murine mortality resulting from salmonellosis. This ability was lost however if the MWCO of the dialysis tubing was reduced to1 kDa (Fig. 6.1).

The thermostability of the bioactive small weight material was assessed by incubating it as an aqueous solution at 85 or 100°C. The resulting material lost bioactivity (Fig. 6.2), which was also destroyed if the aqueous solution was subjected to proteolytic degradation by proteinase K and bromelain (Fig. 6.3). As a consequence of these observations, all subsequent studies employed methodologies consistent with the purification and detection of proteinaceous material.

Size exclusion chromatography was performed on goat serum with a Superdex 200 HR 10/30 column (Fig. 6.4a). Three peaks were detected at an absorbance of 280nm. The first peak (21.5 min) had an estimated molecular mass of approximately 300 kDa and constituted less than 10% of the total integrated area. The other two peaks (23.9 min and 26.3 min) were estimated to have molecular weights of 149 and 68 kDa, and constituted 41 and 51% of the total integrated area, respectively. The majority of the protein present in the goat serum migrated in a manner consistent with immunoglobin and albumin when resolved by SDS gel electrophoresis (data not shown).

The bioactive low molecular weight material obtained after equilibrium dialysis (CSF-I2) was concentrated and resolved subsequently by a Superdex 200 HR 10/30 size exclusion chromatography system. The relative area of the immunoglobin and albumin peaks were greatly reduced and two peaks with molecular weights of 4.9 and 1.7 kDa appeared (Fig 6.4b). When this material was passaged through an Amicon Centriprep YM-10 ultrafiltration device (MWCO 10 kDa), the effluent (i.e., TPI) had no high MW components present; over 90% of the material eluted with a molecular mass of 5.8 kDa (Fig. 6.4c). Bioactivity remained associated with the low molecular mass material (Fig. 6.5). If the material was fractionated with an Amicon Centriprep YM-3 ultrafiltration device (MWCO 3,000), the effluent did not possess bioactivity (data not shown). When TPI (the bioactive low molecular weight material) was resolved by SDS gel electrophoresis only one band with a MW of approximately 6.5 kDa was detected (Fig 6.6).

TPI was lyophilized to a powder and forwarded to Commonwealth Biotechnologies, Inc for resolution by analytical size exclusion chromatography (TSK G2000 SWXL). Material which had resolved as a single peak from a Superdex 200 HR 10/30 column now generated three discernable peaks with estimated molecular weights of 4.2, 1.9, and 1.2 kDa (Fig. 6.7). Peak fractions were combined following multiple analytical fractionations (n=16), concentrated by lyophilization, and tested for bioactivity. A total of 8.8, 22.4, and 35.3 g was obtained for fractions 25, 27, and 29, respectively. Each treated mouse (n=25) received the following: fraction 25 (352 ng/mouse), fraction 27 (896 ng/mouse), and fraction 29 (1,412 ng/mouse). Only fraction 27 appeared to induce ($p = 0.1114$) a reduction in mortality by day 8 (Fig. 6.8).

An analysis of the amino acid composition showed that this fraction was composed of nearly 26% arginine (Table 6.1).

Previous attempts to purify preparatively the bioactive peptidic isolates present in TPI by C-18 reverse phase chromatography were unsuccessful (data not shown). Bioactivity was lost when TPI was exposed to the organic solvents used to resolve the peptides (i.e., 0.1% trifluoroacetic acid, 35% acetonitrile). Analytical C-18 reverse phase chromatography confirmed however the presence of three peaks (Fig. 6.9), which when scanned from 200-400nm revealed a characteristic peptide profile lacking aromaticity (Fig. 6.9 insert).

DISCUSSION

Clinical studies provided evidence for the existence of a goat serum factor that enabled animals to better withstand potentially lethal pathogenic challenges (Willeford *et al.*, 2000; Willeford *et al.*, 2001; Hamm *et al.*, 2002). The physical nature of this factor was unknown. Immunoglobin, a major constituent of serum, had previously been shown to inhibit sepsis in postoperative patients (Duswald *et al.*, 1980). Caprine immunoglobin was unable, however, to inhibit salmonellosis when initiated in accordance with the animal model developed for this study (Willeford *et al.*, 2001). Lipids, carbohydrates, and proteinaceous material are all known to be present in serum. All three of these broad classes of biological compounds have been observed to produce functional immunomodulators (Hadden, 1993; Abbas and Janeway, 2000; Hamm *et al.*, 2002). The caprine serum factor was classified preliminarily as proteinaceous after a

thermal stability study revealed that bioactivity could be eliminated upon incubation at 85°C. This hypothesis was verified when a loss of bioactivity was observed after implementing proteolytic digestion as an assay pretreatment of the serum isolate.

To date, the only proven assay denoting the factor s bioactivity is derived from animal model studies. While reliable, it must be considered insensitive due to the number of animals required to generate observational significance. Purification procedures were employed which permitted initially the large scale or bulk separation of serum components (i.e, proteins versus peptides). This approach helped ensure sufficient availability of assay material while characterizing the factor by its broad isolation classification. A series of size exclusion procedures produced a bioactive sample that while void of proteins (e.g., immunoglobin and albumin) showed the presence of at least three peptides (Figure 6.7). Each peptidic isolate was assayed for bioactivity. Only one of these peptides, fraction 27 (Fig. 6.8), appeared ($p = 0.1114$) to provide a health benefit. Mortality was lessened by as much as 20%. It may be possible to establish greater significance by providing the mice with more of the peptide isolate. The mice in this study received approximately 1 g of peptide, and this was generated by combining fractions from 16 size exclusion chromatography isolations. This provides an indication as to the relative amount of this factor in serum. Further, the stability of this factor throughout purification remains unclear. It is a common occurrence for peptides to become unstable when removed from their physiological milieu - thus, the more naked the peptide, the greater the possibility for denaturation (Harris, 1989).

The bioactive peptide(s) had a molecular weight between 3 and 10 kDa, as determined by molecular filtration devices. When SDS gel electrophoresis was used to resolve bioactive samples the presence of a peptide band with a MW of approximately 6.5 kDa was revealed (Fig. 6.6). Size exclusion chromatography then narrowed the MW range to 4-6 kDa (Fig. 6.7).

It has not been determined whether functional immunomodulation rests with one or more of the peptides isolated. Three factors limited our ability to attack this question successfully, the lack of a sensitive bioassay, the limited abundance of the peptide factor, and the probable denaturation of the peptide(s) of interest upon purification. It may be possible to obtain greater amounts of the bioactive peptide(s) by using a preparative size exclusion column (e.g., BioGel P2). Upon confirming the presence of bioactivity, it may be possible for sequencing to be performed on the peptides of interest, and then through *de novo* synthesis acquire enough purified material to verify bioactivity through an established animal model. Ultimately, it will be essential to elucidate the factor s sequence.

Table 6.1. Amino acid analysis[1].

Amino acid	Mole %		
	Fraction 25	Fraction 27	Fraction 29
As(x)	13.49	7.53	10.96
Gl(x)	9.36	6.31	9.00
Ser	4.41	2.97	4.45
His	2.49	1.01	1.33
Gly	10.66	19.11	8.91
Thr	5.24	4.10	4.84
Ala	12.14	9.04	13.11
Arg	5.05	25.83	6.58
Tyr	3.18	1.78	2.94
Cystine	0.00	0.00	0.46
Val	6.54	3.86	5.95
Met	2.03	1.54	1.84
Trp	NA[2]	NA	NA
Phe	3.48	2.15	3.17
Ile	4.45	2.80	3.63
Leu	7.97	4.94	7.38
Lys	5.35	3.17	8.65
Pro	4.16	3.86	6.80

[1]Aliqouts obtained from the peak fractions resolved by TSK G2000 SWXL size exclusion chromatography were dried and hydrolyzed by gas phase HCl for 20 h at 110°C. Following hydrolysis, the samples were dried, suspended in 0.4M borate (pH 10.2) and derivatized. Primary amino acids were derivatized with orthophthaladehyde and secondary amino acids were derivatized with 9-fluorenylmethoxycarbonyl. Derivatives were separated by reverse phase HPLC and detected by UV absorbance. Quantitation was done with the Aminoquant software (Agilent Technologies) calibrated with Agilent standards.
[2]Not analyzed.

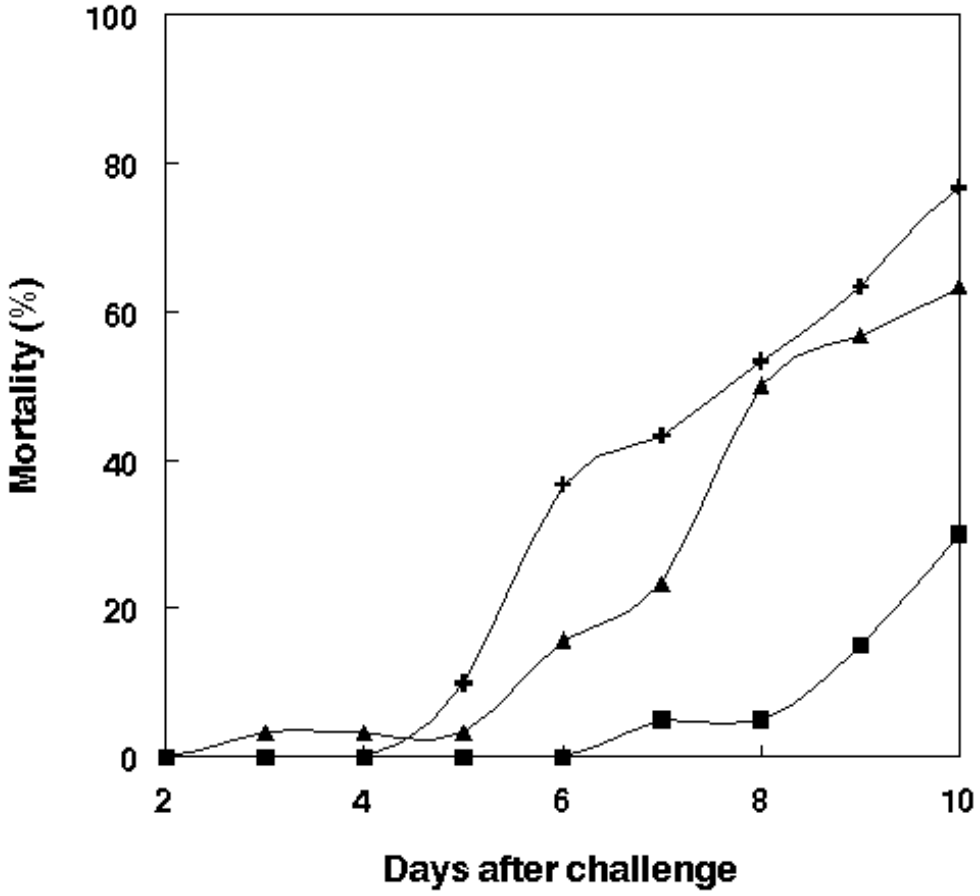

Figure 6.1. Dialysis of caprine serum.

Control mice were administered 0.1 ml (~5 * 10^3 CFU) of *S. typhimurium* i.p. on day 0 (+), while treated mice received both *S. typhimurium* on day 0 and a 0.25 ml subcutaneous injection of 5 mg 6-8 kDa MWCO effluent ( ) or 1 kDa MWCO effluent ( ). Each data point represents the average daily mortality (n = 6) with its associated standard experimental error per cage of 5 mice.

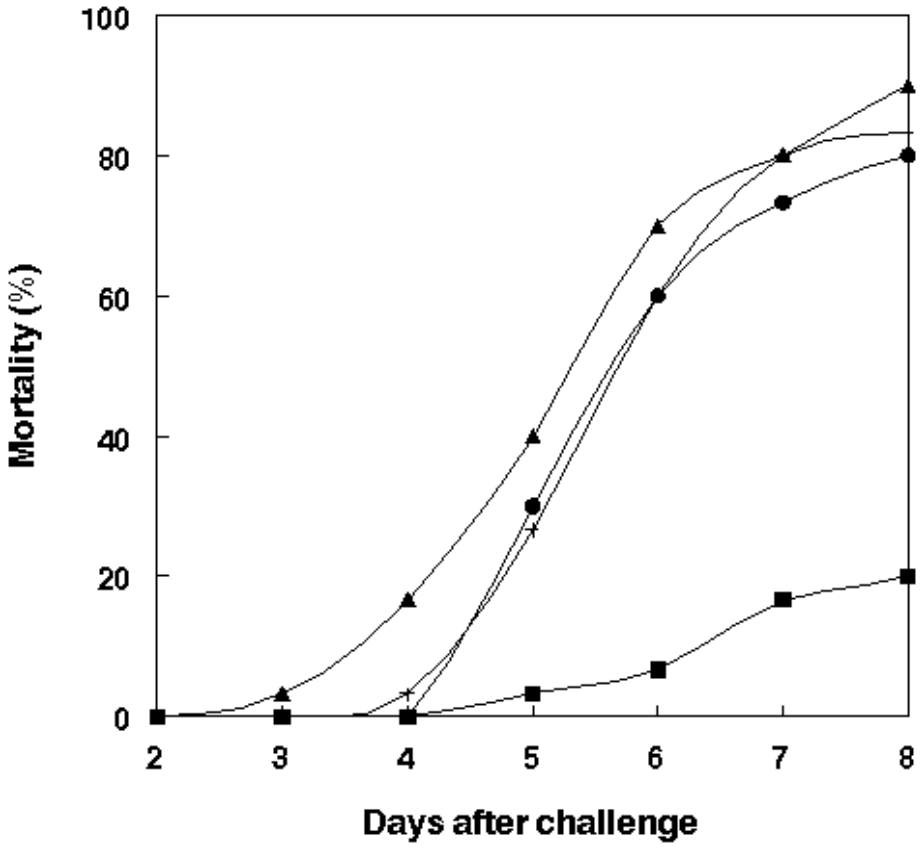

Figure 6.2. Heat denaturation study.

Control mice were administered 0.1 ml (~5 x 10^3 CFU) of *S. typhimurium* i.p. on day 0 (+), while treated mice received both *S. typhimurium* on day 0 and a 0.25 ml subcutaneous injection of 5 mg 6-8 MWCO dialysis effluent ( ) or 5 mg effluent incubated for 30 min at 85°C ( ) or 15 min at 100°C ( ). Each data point represents the average daily mortality (n = 6) with its associated standard experimental error per cage of 5 mice.

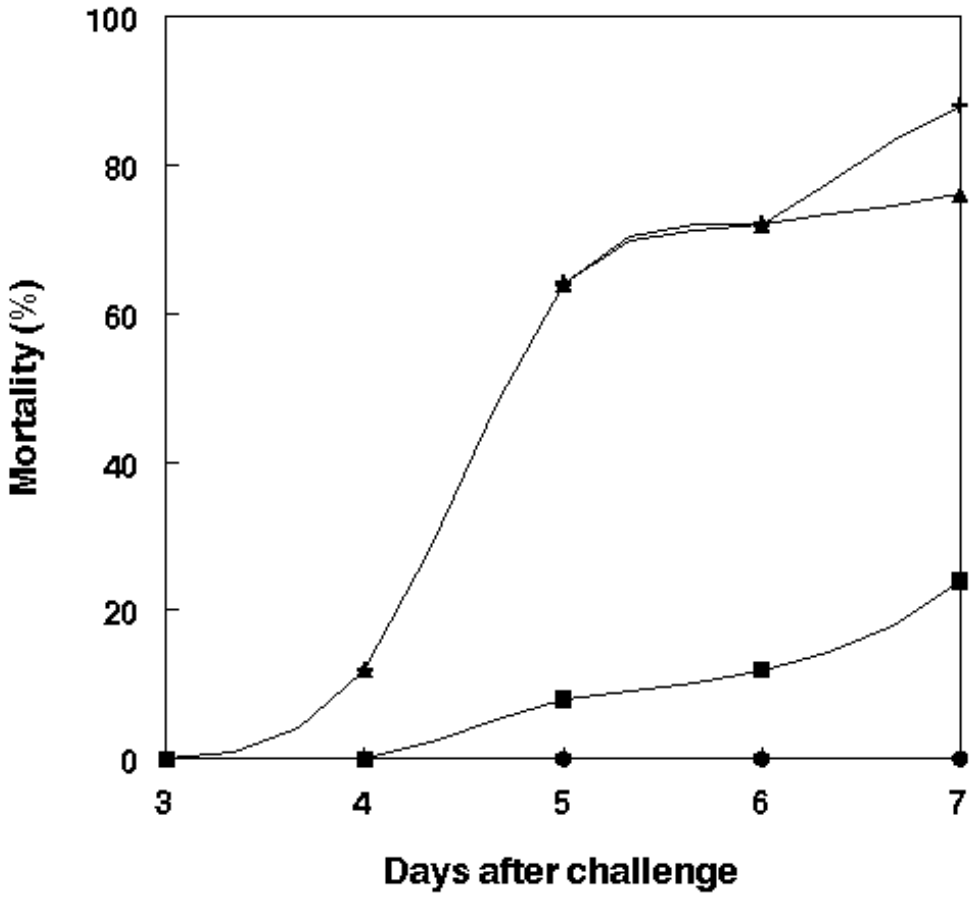

Figure 6.3. Protease denaturation study.

Control mice were administered 0.1 ml (~5 x 10^3 CFU) of *S. typhimurium* i.p. on day 0 (+), while treated mice received both *S. typhimurium* on day 0 and a 0.25 ml subcutaneous injection of 5 mg 6-8 MWCO dialysis effluent ( ) or effluent exposed to bromelain and proteinase K ( ). Positive control mice received only the bromelain/proteinase K cocktail ( ). Each data point represents the average daily mortality (n = 6) with its associated standard experimental error per cage of 5 mice.

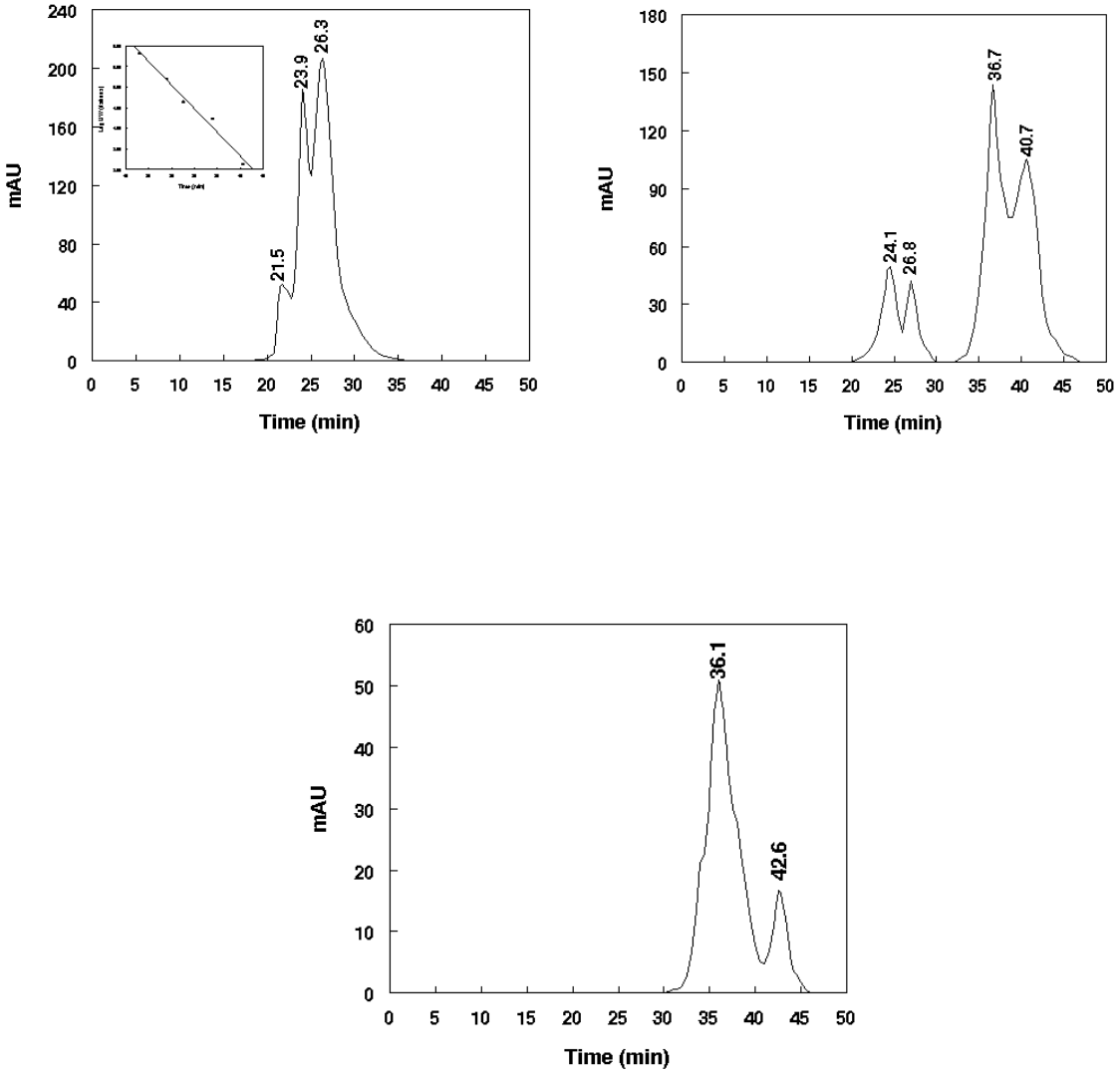

Figure 6.4. Size exclusion chromatography of caprine serum and fractionations.

Twenty 1 aliquots were applied to a Superdex 200 HR 10/30 size exclusion column (Pharmacia LKB Biotechnology) equilibrated in 10 mM sodium phosphate (pH 7.1) and 150 mM NaCl. The column was calibrated with thyroglobulin (670 kDa), IgG (150 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and Vitamin B_{12} (1.35 kDa). The operating system used was a Hewlett Packard 1100 HPLC operated at a flow rate of 1 ml/min and set to detect at 280 nm. Samples run were: a. caprine serum; b. 6-8 kDa MWCO dialysis effluent (10 mg/ml); and c. TPI (10 mg/ml).

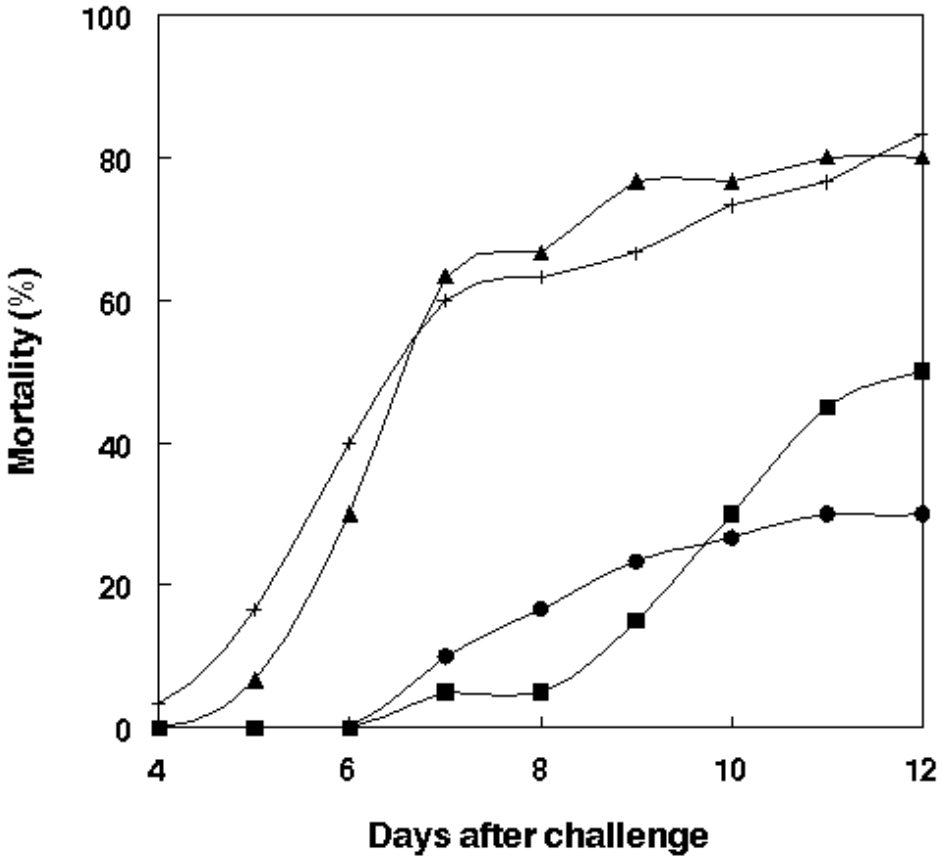

Figure 6.5. Centriprep effluent vs. retentate.

Ultrafiltration was conducted on reconstituted 6-8 MWCO dialysis effluent (10 mg/ml) with an Amicon Centriprep YM-10 ultrafiltration device and the Centriprep retentate and effluent were collected. Control mice were administered 0.1 ml (~5 x 10^3 CFU) of *S. typhimurium* i.p. on day 0 (+), while treated mice received both *S. typhimurium* on day 0 and a 0.25 ml subcutaneous injection of: 5 mg 6-8 kDa MWCO dialysis effluent ( ), Centriprep retentate ( ), or Centriprep effluent ( ). Each data point represents the average daily mortality (n = 6) with its associated standard experimental error per cage of 5 mice.

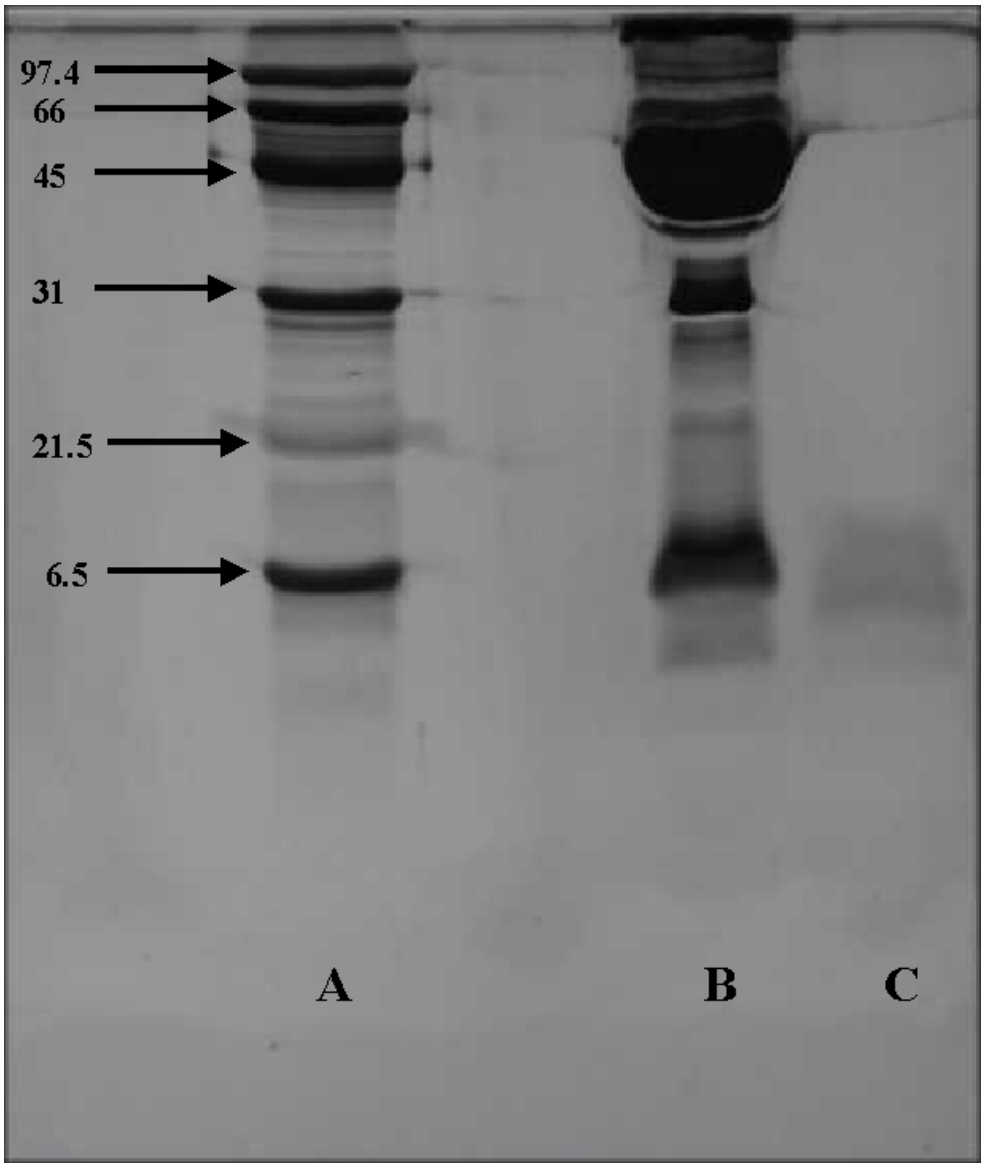

Figure 6.6. SDS gel electrophoretogram of dialysis effluent and TPI.

Dialysis effluent from 6-8 kDa MWCO dialysis tubing and TPI were analyzed on a 15% acrylamide SDS electrophoresis gel and visualized with silver stain. Samples loaded were as follows: Lane A. 10 l BioRad low MW standards (MW in kDa); Lane B. 70 g dialysis effluent; and Lane C. 70 g TPI.

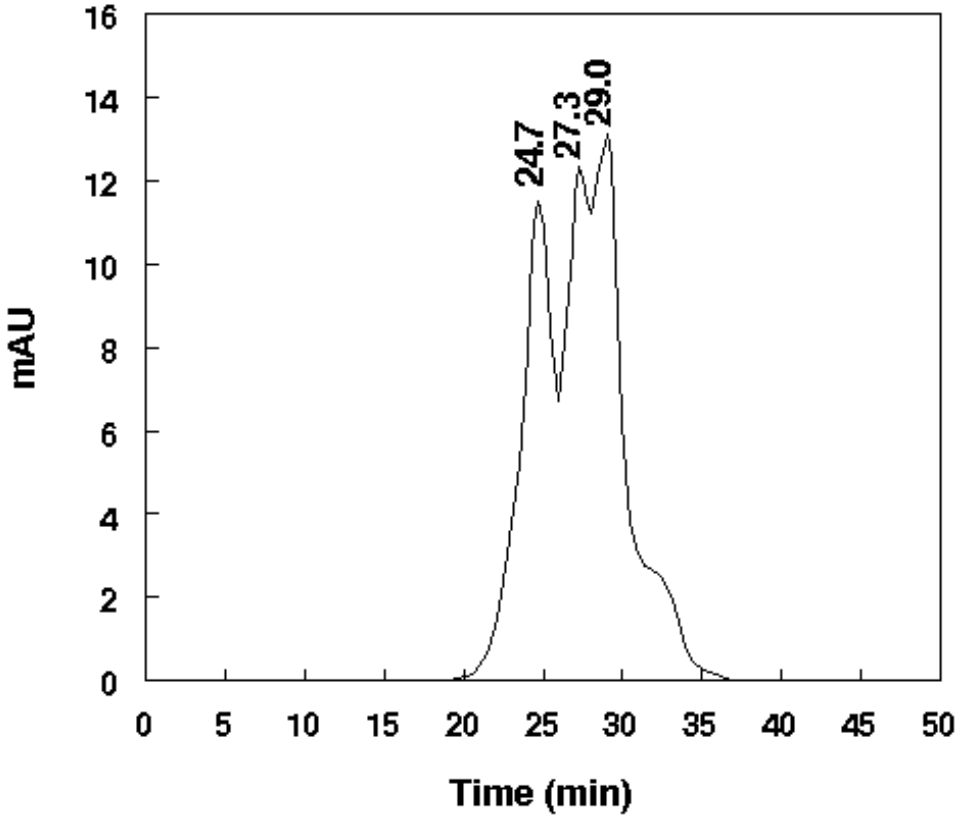

Figure 6.7. Analytical size exclusion chromatography of TPI.

Twenty-five 1 TPI (10 mg/ml) was applied to an analytical TSK G2000 SWXL size exclusion column equilibrated with 200mM potassium phosphate (pH 6.8) containing 15mM NaCl. The system was operated at 0.5 ml/min, the eluate was monitored at 214nm, and 0.5 ml fractions were collected.

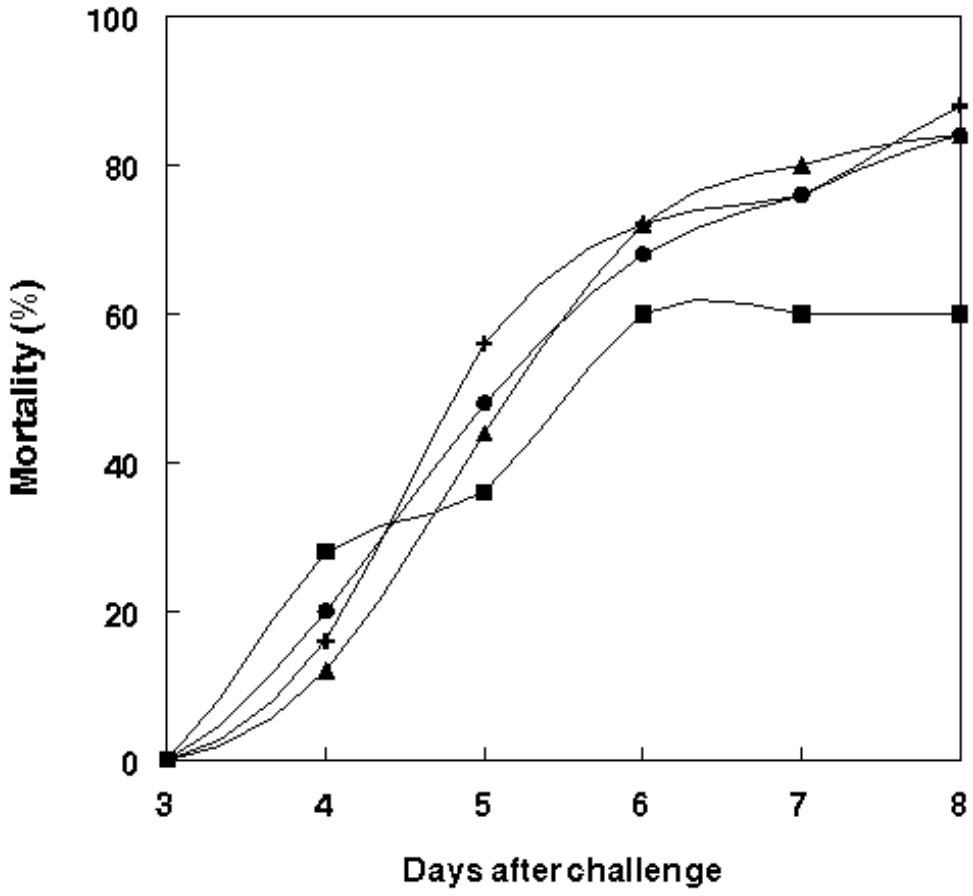

Figure 6.8. Bioactivity of analytical size exclusion fractions.

Control mice were administered 0.1 ml (~5 * 10^3 CFU) of *S. typhimurium* i.p. on day 0 (+), while treated mice received both *S. typhimurium* on day 0 and proteinaceous material obtained from either fraction 25 (352 ng,), 27 (896 ng,), or 29 (1,412 ng,). Each data point represents the average daily mortality (n = 5) with its associated standard experimental error per cage of 5 mice.

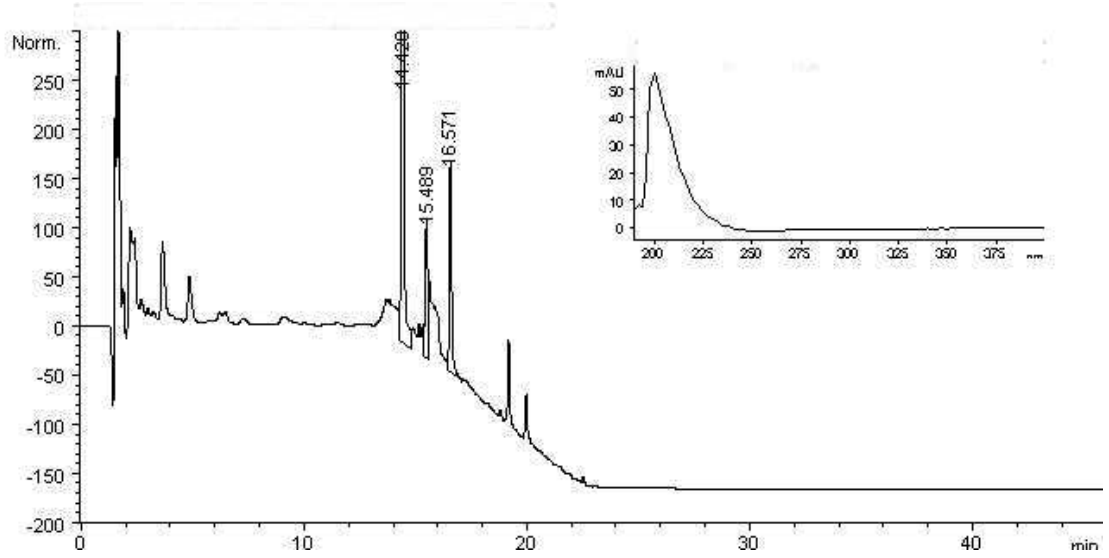

Figure 6.9. C-18 reverse phase chromatography of TPI.

Reverse phase HPLC was performed with a C-18 HPLC column (Alltech Absorbosphere C-18 5U, 150 mm x 4.6 mm) equilibrated in 0.1% aqueous TFA (trifluoroacetic acid). The column (attached to a Waters 600 E HPLC) was washed with this buffer for 10 min (1 ml/min) and bound material eluted by establishing a binary gradient which linearly increased the secondary buffer (80% acetonitrile, 0.1% aqueous TFA) to 100% over a 20 min period. A 100 l aliquot of TPI in 0.1% TFA was applied to the column. Sample elution was monitored at 220 nm with an Agilent 1100 Series diode array detector, and peak fractions were spectrophotometrically scanned from 200-400nm.

CHAPTER VII

CONCLUSION

Controlled studies demonstrated that CSF-I, material fractionated from caprine serum, appeared to possess an immunomodulatory compound (Hamm *et al.*, 2002). Caprine serum was further fractionated into three peptidic components -Caprine serum fraction - immunomodulator 2 or CSF-I2. CSF-I2 does not possess antibacterial capabilities (as typically characteristic of a cationic peptide or defensin), does not contain a level of endotoxin sufficient to promote a pyrogenic response, and its functional ability to improve animal survival after an infectious challenge does not reside with molecular weight components greater than 10 kDa, effectively excluding the immunoglobins, albumin, cytokines, and collectins. Benefit appears to derive from proteinaceous components in light of the observation that all benefit is lost after proteolytic digestion with bromelain and proteinase K or incubation at 85°C, procedures known to denature protein.

CSF-I2 was able to significantly reduce the mortality observed in chickens (from 80% to 13%) infected with *Pasteurella multocida* (Willeford *et al.*, 2000), in mice (from 83% to 13.3%) infected with *Salmonella typhimurium*, and in canines (from 50% to 9.8%) diagnosed with parvovirus. CSF-I2 may effectively combat pathogenesis when

used as either an adjunct with conventional therapy (e.g., antibiotics) or when provided as the primary medicant. CSF-I2 may well prove to provide prophylactic and therapeutic health benefits to humans.

The reduction in mortality realized by pretreatment with CSF-I2 may be due to an enhanced clearance of *Salmonella* in the peritoneal cavity, thereby retarding bacterial dissemination and inflammation. A reduction in inflammation was noted to occur in chickens infected with *Pasteurella multocida* as evidenced by CSF-I2 treated chickens expressing lower heterophil counts and increased CD4 counts. Our studies also showed that a caprine serum factor reduced microbial proliferation in splenic tissue.

While CSF-I2 does not appear to work through directly stimulating macrophages, it remains possible that CSF-I2 primes macrophages for augmented production of pro-inflammatory cytokines, e.g., in response to *S. typhimurium*. IFN- and IFN- / have been shown to control the growth of intracellular pathogens by inducing macrophage production of and subsequent response to interferons (Rothfuchs *et al.*, 2001). This action would effectively limit the replication of *Salmonella* at the early stage of infection and may allow neutrophils and NK cells to more adequately restrict the systemic spread of *S. typhimurium* and prolong the survival of mice challenged with a potentially lethal dose.

It has not been determined whether functional immunomodulation rests with one or more of the peptides isolated, although one peptide appeared to be active. Three factors limited our ability to attack this question successfully, the lack of a sensitive bioassay, the limited abundance of the peptide factor, and the probable denaturation of

the peptide(s) of interest upon purification. It may be possible to obtain greater amounts of the bioactive peptide(s) by using a preparative size exclusion column (e.g., BioGel P2). Upon confirming the presence of bioactivity, it may be possible for sequencing to be performed on the peptides of interest, and then through *de novo* synthesis acquire enough purified material to verify bioactivity through an established animal model. Ultimately, it will be essential to elucidate the factor s sequence.

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